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Molecular Basis of Paralytic Neurotoxin Action on Voltage-Sensitive Sodium Channels

Annual Report

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In years 1 and 2 of this project, progress was made on several objectives:

1. The sites and mechanisms of action on the sodium channel were examined and further defined for three new classes of neurotoxins: Goniopora toxins, Brevetoxins, and Conotoxins.
2. Monoclonal antibodies with high affinity for the mammalian neuronal sodium channel were developed and methods to screen them for activity at neurotoxin binding sites were established.
3. Site-directed antibodies against defined regions of the amino acid sequence of the sodium channel were prepared and shown to bind at discrete negatively charged subsites on the extracellular surface of the channel that may form part of neurotoxin receptor sites.

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Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Research Report

In the second year of this project, we have continued to analyze the effects of newly described neurotoxins on voltage-sensitive sodium channels in mammalian neurons, developed a panel of monoclonal antibodies against sodium channels of mammalian neurons, and prepared site-directed antisera against several defined segments of the sodium channel.

A. Neurotoxin Action on Sodium Channels

1. Site and Mechanisms of Action of Goniopora Toxin.

The results on the site and mechanism of action of Goniopora toxin on sodium channels described in last year's Annual Report (October 14, 1985) were completed and have now been published in *Molecular Pharmacology*.

2. Inhibition of Sodium Channels by μ-Conotoxins.

Most species belonging to the gastropod family Conidae use venoms in the capture of prey organisms. Among them, Conus geographus is most toxic to vertebrates and has been responsible for human fatalities (3). The venom of C. geographus causes complete loss of contractile responses of skeletal muscles (4-6). Recently, three classes of peptide toxins have been isolated and their structures determined. Conotoxins GI, GIA and GII are blockers of nicotinic acetylcholine receptors (7); ω-conotoxin blocks neuronal calcium channels (8, 9); and geographutoxins (GTX)I and II (10, 11), also called μ-conotoxins (12), have the novel action of blocking sodium channels in muscle much more effectively than those in nerve (12, 14). Our results show that this novel action may be mediated by interaction of the peptide toxin with the tetrodotoxin/saxitoxin receptor site on the sodium channel providing the first evidence that toxins can distinguish between the tetrodotoxin/saxitoxin receptor sites on nerve and muscle sodium channels.

Concentration dependence of inhibition of muscle contraction by GTX II. The primary structure of the novel polypeptide toxin GTX II is illustrated in Figure 1. The presence of three hydroxyproline residues and 6 cysteine residues in disulfide bonded form within the 22 residue sequence distinguish the structure GTX II and its congeners (10-12) from other polypeptide toxins that have been characterized. In order to make a quantitative estimate of $K_{0.5}$ values for inhibition of muscle contraction by GTX II, the effects of a range of toxin concentrations on contractile responses of rat and rabbit diaphragm muscle to direct electrical stimulation were measured. The resulting concentration-effect curves (Figure 2) show that GTX II inhibits contraction in rat and rabbit muscle with $K_{0.5}$ values of 60 nM and 50 nM, respectively. These values are similar to the apparent K_D of 52 nM measured for block of muscle sodium channels in planar bilayers by the related conotoxin GIIIA by Cruz et al (12). These indirect estimates of toxin binding affinity in physiological assays provide a standard of comparison for direct binding studies.

Site of GTX II action. Of the numerous neurotoxins that act on sodium channels, only tetrodotoxin and saxitoxin block sodium channels rapidly and reversibly as observed for GTX II and its congeners (12-14). Moreover, inhibition of batrachotoxin-activated sodium channels by GTX II is voltage-dependent (12) as for saxitoxin and tetrodotoxin. These similarities suggest the possibility that GTX II, tetrodotoxin and saxitoxin may act at the same receptor site.

Previous studies have shown that tetrodotoxin and saxitoxin bind at a common receptor site on sodium channels that has been designated neurotoxin receptor site 1 (reviewed in 2, 13, 14). Measurement of specific binding of [^{3}H]saxitoxin provides a direct measure of toxin interaction with that site (2, 13, 14). Figure 3 illustrates the inhibition of specific saxitoxin binding to neurotoxin receptor site 1 on sodium channels in a total rat muscle homogenate or in purified rabbit transverse tubules. [^{3}H]saxitoxin binding is blocked completely with half-maximal inhibition at 60 nM in whole homogenate and 35 nM in purified transverse tubules. These $K_{0.5}$ values are similar to those for inhibition of muscle contraction in agreement with the conclusion that GTX II inhibits sodium channels through interaction with neurotoxin receptor site 1.

The dependence of [^{3}H]saxitoxin binding on free toxin concentration in the presence and absence of 50 nM GTX II illustrated in Figure 4A. Specific binding was determined from the difference between total binding and the fitted line for nonspecific binding measured in the presence of 2 μM tetrodotoxin. Scatchard analysis showed that saxitoxin binds to a single class of high affinity receptor sites with K_D = 9.3 nM at 36°. GTX II (50 nM) increased the K_D for [^{3}H]saxitoxin from 9.3 to 25.3 nM. The maximal binding capacity was slightly reduced from 300 to 240 fmol/mg protein. These results indicate a primarily competitive mode of interaction between GTX II and saxitoxin consistent with interaction of GTX II at neurotoxin receptor site 1 with K_D values of 24 nM and 35 nM for sodium channels in T-tubules or homogenates, respectively.

Tissue specificity of GTX II binding at neurotoxin receptor site 1. The physiological effects of GTX II and its congener conotoxin GIIIA on sodium channels are highly tissue specific. No inhibition of batrachotoxin-activated sodium channels from brain membranes incorporated into planar lipid bilayers is observed at 1 μM (unpublished observations). Figure 5 illustrates the effect of GTX II on [^{3}H]saxitoxin binding to rat brain synaptosomes and rabbit superior cervical ganglion membranes. Saxitoxin binding to these neuronal membrane preparations is unaffected by 35 nM GTX II, a concentration which gives 50% inhibition of toxin binding to muscle membranes. Higher concentrations of GTX II inhibit saxitoxin binding to synaptosomes with an EC_{50} of 2 μM, but reduce binding to superior cervical ganglion membrane only 10% at 10 μM. These results show that the marked tissue specificity in the actions of GTX II and conotoxin GIIIA may derive from marked differences in toxin binding affinity at neurotoxin receptor site 1 on the sodium channel.

Three other neurotoxin receptor sites associated with the sodium channel have been defined in neurotoxin binding studies in synaptosomes and other neural tissues (reviewed in 1, 13). We have examined GTX II interaction with two of these. GTX II has no effect on binding of [^{3}H]BTX-B to neurotoxin receptor site 2 at concentrations ranging from 10 nM to 10 μM (data not shown). Similarly, GTX II has no effect on binding of ^{125}I-labeled scorpion toxin to neurotoxin receptor site 3 over the same concentration range (data not shown).

GTX II acts at neurotoxin receptor site 1 on the sodium channel. Our results provide strong evidence that GTX II inhibits sodium channels by interaction with neurotoxin receptor site 1 or a physically overlapping site. Binding of saxitoxin at that site is competitively inhibited by GTX II with a K_D approximately equal to the $K_{0.5}$ for block of muscle contraction and muscle sodium currents (10-12). Inhibition of saxitoxin binding to sodium channels in neuronal membranes requires much higher concentrations of GTX II in agreement with the lack of inhibition of sodium channels in neurons by this toxin. An allosteric mechanism of inhibition is unlikely because both saxitoxin and GTX II inhibit sodium channels via a similar mechanism with similar voltage dependence indicating that they bind to the same functional states of sodium channels. If the two

toxins did bind to separate, allosterically interacting sites, they would be expected to bind and act synergistically.

The structure of the polypeptide toxin GTX II (Figure 1) is quite different from that of tetrodotoxin and saxitoxin which are low molecular weight heterocyclic guanidines. It is likely that it occupies a larger binding surface than tetrodotoxin and saxitoxin. The guanidine moieties of the multiple arginine residues of GTX II may allow it to interact with neurotoxin receptor site 1 directly in addition to other nearby regions of the extracellular surface of the sodium channel.

Sodium channels in nerve and adult skeletal muscle differ in molecular properties. Initial comparisons of the physiological and pharmacological properties of sodium channels in nerve and adult skeletal muscle revealed many similarities and no important differences (15). In contrast, more recent work has revealed striking pharmacological and molecular differences. Crotamine, a polypeptide from certain rattlesnake venoms, blocks muscle sodium channels more effectively than nerve (16). Polyclonal antisera against brain sodium channels crossreact poorly with muscle channels (17). Muscle sodium channels have a 20% higher single channel conductance than nerve channels (18). These results support the view that skeletal muscle and neuronal sodium channels are different molecular species. Our results provide more definitive evidence for this conclusion by showing that GTX II differs sharply in its interaction with neurotoxin receptor site 1, a discrete functional region of the nerve and muscle sodium channels. While these differences might result from posttranslational modification, it seems more likely that they reflect differences in primary structure of the two channels. It will be of considerable interest to use GTX II to identify these tissue-specific regions of sodium channel structure at neurotoxin receptor site 1. These results have been published in the *Journal of Biological Chemistry* (46).

3. Site of Action of Brevetoxins.

The Florida red tide dinoflagellate Ptychodiscus brevis produces multiple nonprotein toxins that have been purified to homogeneity including PbTx-1 (also designated T46, GB-1, or brevetoxin A, references 19-21), PbTx-2 (also designated T47, GB-2, T34 or brevetoxin B, references 19, 22-23), and PbTx-3 (also designated GB-3 or T17, references 20, 24). PbTx-1 (21) and PbTx-2 (23) have been determined by X-ray crystallography to be distinct, ladderlike structures of 10 or 11 fused ether rings. PbTx-3 can be produced by reduction of the aldehyde moiety of PbTx-2 to a primary alcohol.

Pure preparations of of PbTx-1 enhance persistent activation of sodium channels in mouse neuroblastoma cells at concentrations consistent with its toxic actions on fish and mice (25, 26). PbTx-2 and -3 each depolarize squid and crayfish axons under voltage clamp by causing prolonged activation of sodium channels (27). The voltage dependence of channel activation is shifted 35 mV to more negative membrane potentials and inactivation is blocked (27). These results establish the sodium channel as an important receptor site for the toxic actions of the pure brevetoxins.

The sodium channel has multiple receptor sites for neurotoxins (reviewed in references 13, 14, 28). Tetrodotoxin and saxitoxin bind at neurotoxin receptor site 1 and inhibit ion conductance. Grayanotoxin and the alkaloids veratridine, batrachotoxin, and aconitine bind to neurotoxin receptor site 2 and cause persistent activation of sodium channels. Polypeptide α-scorpion toxins and sea anemone toxins bind at neurotoxin receptor site 3, slow sodium channel inactivation, and enhance persistent activation of sodium channels by toxins acting at site 2 via an allosteric mechanisms. β-scorpion toxins bind at neurotoxin receptor site 4 and alter sodium channel activation. We have

analyzed the effects of PbTx-2 on toxin binding at each of these four neurotoxin receptor sites and established that PbTx-2 enhances toxin binding at sites 2 and 4 via an allosteric mechanism.

Neurotoxin receptor site 1. The interaction of the sodium channel blockers tetrodotoxin, saxitoxin, and geographutoxin II, a toxin from cone snails, with neurotoxin receptor site 1 on sodium channels in rat brain synaptosomes can be monitored by measurement of specific binding of [^{3}H]saxitoxin (29). Figure 6 illustrates the effect of PbTx-2 on specific [^{3}H]saxitoxin binding. No effect is observed at concentrations between 1 nM and 1 μM. Since PbTx-2 modifies sodium channel properties in squid giant axon in this concentration range (27), the results indicate that it has no effect on ligand interaction with neurotoxin receptor site 1.

Neurotoxin receptor site 2. The interaction of the sodium channel activators batrachotoxin, veratridine, aconitine, and grayanotoxin with neurotoxin receptor site 2 on sodium channels in rat brain synaptosomes can be monitored by measurement of specific binding of [^{3}H]BTX-B (30). However, it can be markedly enhanced by LqTx through allosteric interactions at neurotoxin receptor site 3 (30). Figure 7 shows that PbTx-2 increases specific binding of [^{3}H]BTX-B 2.9-fold with half maximal effect at 20 nM. Evidently PbTx-2, like LqTx, can enhance [^{3}H]BTX-B binding through allosteric interactions.

The effects of PbTx-2 and LqTx on [^{3}H]BTX-B binding are synergistic. Figure 8 illustrates the time course of [^{3}H]BTX-B binding in the presence of saturating concentrations of PbTx-2 (1 μM) alone or PbTx-2 and LqTx (1 μM). The time course of binding is similar approaching equilibrium in 60 min at 36°. However, the equilibrium binding of [^{3}H]BTX-B is increased 3.1 fold over that observed with PbTx alone. Table 1 summarizes the levels of specifically bound [^{3}H]BTX-B observed in the absence of other neurotoxins or in the presence of saturating concentrations of PbTx-2, LqTx, or a combination of the two toxins. The two toxins, when present together, cause an enhancement of [^{3}H]BTX-B binding that is more than the sum of the effects of PbTx-2 and LqTx when present separately. This synergistic interaction suggests that PbTx-2 and LqTx can simultaneously bind at different receptor sites associated with sodium channels and each enhance [^{3}H]BTX-B binding.

The effects of combinations of LqTx and PbTx-2 on K_D and B_{max} values for specific binding of [^{3}H]BTX-B were studied by Scatchard analysis of the concentration dependence of toxin binding (Figure 9). No significant changes in B_{max} were observed. The K_D in the presence of LqTx alone is 116 nM in reasonable agreement with earlier work (20). The K_D in the presence of PbTx-2 (270 nM) is higher than in the presence of LqTx as expected from the lower levels of binding observed at a fixed concentration (10 nM) of [^{3}H]BTX-B (Table 1). In the presence of both agents, the K_D for BTX-B is reduced to 68 nM. These changes in K_D are consistent with the synergistic effects of PbTx-2 and LqTx on BTX-B binding at 10 nM [^{3}H]BTX-B although a quantitative comparison is not possible because the low level of specific binding observed in the absence of LqTx and PbTx-2 prevents an accurate measurement of K_D.

Neurotoxin receptor site 3. The interaction of polypeptide neurotoxins from scorpion and sea anemone venoms with receptor site 3 on sodium channels can be monitored by measurement of specific binding of [^{125}I]$_1$LqTx (29). In order to test directly whether PbTx-2 affects toxin binding and action at site 3, its effects on specific binding of [^{125}I]$_1$LqTx were studied. The results of Figure 6 show that PbTx-2 has no effect on neurotoxin binding at site 3. Evidently, its effects on binding of [^{3}H]BTX-B arise from interaction at a different receptor site than those of LqTx.

Neurotoxin receptor site 4. The interaction of β-scorpion toxins with neurotoxin receptor site 4 on sodium channels can be studied by measurement of specific binding of [^{125}I]CsTx II (31). Figure 10 illustrates the effect of PbTx-2 on CsTx II binding. Specific binding is increased 2.7 fold with a half maximal effect at approximately 30 nM PbTx-2. This effect is similar in magnitude and concentration dependence to the enhancement of [^{3}H]BTX-B binding by PbTx-2 (Figure 7).

Analysis of the effects of PbTx-2 on B_{max} and K_D for [^{125}I]CsTx II is illustrated in the form of a Scatchard plot in Figure 11. The K_D is decreased 2.5 fold from 5.2 nM to 2.3 nM without a significant change in B_{max}. Thus, the effect of PbTx to enhance CsTx II binding results from an effect on K_D with no change in B_{max}, like its effect on BTX-B binding.

Voltage dependence of PbTx action. The binding and action of neurotoxins at receptor site 3 on sodium channels is highly voltage-dependent (13, 28), decreasing progressively with membrane depolarization. In order to examine possible voltage dependence of PbTx action in enhancing specific binding of [^{3}H]BTX-B, synaptosomes were incubated with [^{3}H]BTX-B and a concentration of PbTx-2 (30 nM) near its apparent K_D, in the presence of varying concentrations of K^+ to alter the resting membrane potential. Figure 12 compares the effect of increased external K^+ on the binding of ^{125}[I]$_1$LqTx and on the enhancement of [^{3}H]BTX-B binding. Between the resting membrane potential of synaptosomes at 5 mM K^+ (approximately -55 mV, (23)), and the membrane potential at 135 mM K^+ (approximately 0 mV, (23)), binding of [^{125}I]$_1$LqTx is reduced 8-fold (Figure 7). The enhancement of [^{3}H]BTX-B binding by PbTx-2 is also reduced by depolarization of synaptosomes with elevated external K^+ (Figure 7). The dependence on K^+ concentration is less steep than observed for binding of [^{125}I]$_1$LqTx with a 4-fold change between 5 mM K^+ and 135 mM K^+. The binding of [^{3}H]BTX-B itself is not strongly affected across this voltage range. The results suggest a significant voltage-dependence of PbTx-2 binding and action.

Site of PbTx action. Our results provide clear evidence that PbTx-2 exerts its effects on sodium channels by interaction with a new receptor site not previously described in direct radioligand binding studies. We show that PbTx-2 has no effect on neurotoxin binding at sites 1 and 3 and enhances neurotoxin binding at sites 2 and 4. Thus, it must act at a new neurotoxin receptor site, site 5, associated with sodium channels. This new receptor site has recently been detected in direct binding studies using [^{3}H]PbTx-3 produced by reductive tritiation of PbTx-2 (32). As expected from the results described here, toxins acting at sites 1-4 do not block specific binding of [^{3}H]PbTx-3 providing further evidence of action at a new receptor site.

Previous studies of the actions of a related sodium-channel-specific neurotoxin, PbTx-1 on sodium channels in mouse neuroblastoma cells and rat brain synaptosomes reached the conclusion that PbTx-1 must also act at a new sodium channel receptor site (25, 26). This toxin enhances activation of sodium channels by veratridine, aconitine, and batrachotoxin acting at neurotoxin receptor site 2 without inhibition of binding of saxitoxin or [^{125}I]$_1$LqTx at sites 1 and 3, respectively. Interactions at neurotoxin receptor site 4 have not been studied. PbTx-2 blocks specific binding of PbTx-3 to neurotoxin receptor site 5 on sodium channels consistent with the conclusion that it shares this receptor site for toxin action (32). We expect that PbTx-1 also shares this receptor site although direct experimental evidence for this conclusion is not yet available.

The allosteric modulation of neurotoxin binding and action at sites 2 and 4 by PbTx-1 and PbTx-2 acting at site 5 indicate that there are conformational interactions among these three sites mediated through the sodium channel structure. Allosteric interactions between neurotoxin receptor sites 2 and 3 have been described previously in terms of a model in which neurotoxin action on sodium channels results from selective high affinity binding to individual functional states of sodium channels (33). Neurotoxins acting at receptor sites 2, 4, and 5 have some similar effects on sodium channel function: each of these classes of toxins shifts the voltage-dependence of sodium channel activation to more negative membrane potentials and toxins acting at sites 2 and 5 slow or block sodium channel inactivation (reviewed in 13, 27, 28). These actions suggest that all three of these classes of neurotoxins may act, at least in part, by binding with high affinity to active states of sodium channels and stabilizing them according to the law of mass action, as has been previously shown for the site 2 toxins (33). As the structure of the sodium channel is becoming known in some detail, we are optimistic that the location and structure of these toxin binding sites and the pathways of interaction between them may be elucidated in the future.

B. Production of Monoclonal Antibodies Against the Sodium Channel

The sodium channel is a highly conserved protein which has proven to be a poor antigen in the hands of most investigators. Thus, both polyclonal and monoclonal antibodies prepared against sodium channels from eel electroplax crossreact poorly with sodium channels in nerve and muscle (34, 35). Occasional monoclonal antibodies which do crossreact generally have low affinity (36, but see 37 for an exception). Polyclonal and monoclonal antibodies against sodium channels from rat muscle crossreact poorly with nerve (38, 39). Polyclonal antisera against rat brain sodium channels do not crossreact with rat muscle and actually crossreact with sodium channels in peripheral nerve relatively poorly (40). Antibodies that alter neurotoxin action on sodium channels have been observed in only one case (37). These various studies from several laboratories all lead to the conclusion that antibodies are usually produced against unique tissue- and species-specific epitopes on the sodium channels rather than conserved regions present in all sodium channels. Since the functional elements of sodium channels and the toxin receptor sites which modulate channel function are highly conserved, isolation of antibodies against these regions will require screening of large numbers of independent antibody isolates or use of new experimental approaches.

Antibodies against detergent-solubilized sodium channel. In the first year of work on this proposal, we established methods for production and screening of monoclonal antibodies using both *in vivo* and *in vitro* immunization techniques and isolated the first high affinity monoclonal antibodies directed against sodium channels in mammalian neurons. For *in vivo* immunization, mice were immunized with repeated doses of highly purified sodium channels until a high titer of anti-sodium channel antibodies was produced. Splenic lymphocytes were then dissociated from excised tissue and fused with clone SP2-derived myeloma cells using polyethylene glycol. Replicating hybrid cells were selected in HAT medium and screened for secretion of anti-sodium channel antibodies in a single step using a specific radioimmune assay for sodium channels (41). This single-step screening procedure with highly purified sodium channel reagents was found to be quite important in accurately identifying antibody-secreting clones of interest within a few days after fusion and rapidly cloning them. Using this approach, we have isolated several hydridoma cell lines which secrete a high titer of monoclonal anti-sodium channel antibodies. Representative clones have been injected into mice for production of larger amounts of antibody in ascites fluids. Immunoprecipitation of ^{32}P-labeled sodium channels by ascites fluids from one of these clones (2E8) is illustrated in

Figure 13. Half-maximal immunoprecipitation of sodium channels in our radioimmune assays is produced by 0.006 µl of this ascites fluid.

The affinity of this mAb for sodium channels was estimated using a competition binding assay in which increasing amounts of unlabeled sodium channel were added to the immunoprecipitation reaction to compete with ^{32}P-labeled sodium channel. Half-maximal inhibition of immunoprecipitation is observed with 6 nM unlabeled sodium channel (Figure 14A). Scatchard analysis of these data (Figure 14B) yields a K_D value of 6.4 nM and a binding site concentration in the ascites fluid of 6.8 µM. Thus, this mAb has high affinity and high titer for detergent solubilized rat brain sodium channels.

Antibodies against reconstituted sodium channels. Detergent-solubilized sodium channels lose most of the functional properties of native sodium channels (13, 44). Reconstitution of purified sodium channels from rat brain into phospholipid vesicles of appropriate composition restores all of the functional properties that have been studied to date (13, 44). Since the loss of functional properties on detergent solubilization might be accompanied by changes in antigenic sites we have also developed methods for preparation of antibodies against purified and reconstituted sodium channels. Purified sodium channels were reconstituted in phosphatidylcholine vesicles and injected intraperitoneally into mice using an injection schedule similar to that for detergent solubilized sodium channels. Monoclonal antibodies were isolated as described above using radioimmune assays with both solubilized and reconstituted sodium channels to screen for producing clones. Several hybridoma cell lines were isolated and those producing high affinity antibodies against the sodium channel were selected for further analysis. Large amounts of antibody were produced in ascites fluids in syngeneic mice. Figure 15 illustrates a radioimmunoassay curve for one such representative antibody. Half maximal immunoprecipitation is observed with 0.0001 µl of ascites fluid. The affinity of this monoclonal antibody for sodium channels in synaptosomal membranes was examined using a pre-adsorption assay. Ascites fluid was incubated with increasing amounts of synaptosomes containing a known concentration of saxitoxin binding sites and antibodies bound to sodium channels in synaptosomes were removed by centrifugation. The remaining antibodies in the supernatant were then measured by radioimmune assay with purified sodium channels. Data from this kind of experiment from Ab 3G 4 are illustrated in Figure 16 and 17. Synaptosomal sodium channels reduced the immunoreactivity of the ascites fluid to 50% of maximum at a concentration of 1.0 nM consistent with a K_D value of 1.1 nM. Thus, the affinity of this mAB for native sodium channels is high.

Data for several mAb's that have been characterized to date are collected in Table 3. The K_D values for all the high affinity antibodies we have characterized are in the range of 1 to 20 nM for solubilized and purified sodium channels and in the range of 3 to 25 nM for sodium channels in synaptosomes. Antibody titers in ascites fluids range from 7 to 70 µM. These antibodies are therefore excellent reagents for further studies of sodium channels.

All monoclonal antibodies that have been developed have been tested for inhibition neurotoxin binding at sites 1 through 3 on sodium channels in synaptosomes, [^{3}H]saxitoxin at site 1, [^{3}H]batrachotoxinin A 20-α-benzoate at site 2, and [^{125}I]-labeled α-scorpion toxin at site 3. Representative data are illustrated in Figure 18. Unfortunately, none of the mAb's tested to date has a specific effect on neurotoxin binding to brain sodium channels.

The immune response to conserved regions of proteins is often suppressed by poorly understood mechanisms *in vivo*. It has been suggested that these suppression phenomena

can be avoided if antigen is presented to lymphocytes *in vitro* in cell cultures (42). Thus, *in vitro* immunization may provide an approach to increasing the probability of isolation of antibodies against conserved functional regions of sodium channels. We have now established the *in vitro* immunization method in our laboratory. In several trials of immunization of freshly dissociated cultured lymphocytes by this method (42), we have identified multiple hybridoma clones producing antibodies against the sodium channel, but none of these have proven to have high affinity for the channel. These clones have not been characterized further. We intend to apply this method more extensively in the next year using assay procedures designed to detect high affinity antibodies directed against neurotoxin receptor sites soon after fusion to form hybridomas.

C. Site-directed Antibodies that Alter Neurotoxin Action

We have now determined nearly the complete amino acid sequence of the α subunit of the sodium channel from rat brain by cDNA sequencing methods (43) and Noda et al (45) have reported the complete sequence of two forms of the α subunit. Most of the functional sites of the sodium channel reside on this subunit. We have selected regions of the α subunit that are likely to be components of receptor sites for positively charged sodium channel neurotoxins like saxitoxin and scorpion toxin by identifying regions of concentrated negative charge located on the extracellular surface of the channel. The corresponding polypeptides have been synthesized, covalently coupled to carrier proteins, and injected into rabbits to produce polyclonal antisera. One of these antisera has now been characterized in detail. The binding of peptide SP1 to these antibodies is illustrated in Figures 19 and 20. ^{32}P-labeled sodium channels are bound half maximally at 0.2 μl of antiserum (Figure 19). Saturable binding of [^{125}I]-labeled SP1 is observed in the range of 0 to 5 nM peptide (Figure 20). Analysis of the specific binding data by Scatchard plot yields a K_D of 0.8 nM for SP1.

The affinity of the antipeptide antiserum for the purified sodium channel was determined in a competition binding assay (Figure 21). Binding of ^{32}P-labeled sodium channels was reduced to half of maximum by 2 nM unlabeled sodium channel. Scatchard analysis of these data gives a K_D value of 1.1 nM for purified sodium channels. Thus, this antipeptide antibody has a similar affinity for the native sodium channel.

In order to determine whether these antibodies bind to native sodium channels in intact membranes and to establish whether their binding site is on the intracellular or extracellular face of the membrane, the antiserum was incubated with intact or lysed synaptosomes, bound antibodies were removed by sedimentation, and the remaining unbound antibodies in the supernatant were quantitated by radioimmune assay. These binding data are illustrated as a Scatchard plot in Figure 22. Antibody against SP1 binds to sodium channels in synaptosomes with a K_D of 13.2 nM. Thus, the affinity for the native sodium channel in intact synaptosomal membranes is 10-fold lower than for purified sodium channels or SP1 peptide. Evidently, this antigenic site is accessible in the native sodium channel but antibody access is hindered relative to solubilized preparations.

Figure 23 compares the binding of antibodies against peptide SP1 to sodium channels in intact and lysed synaptosomes. Lysis reduces scorpion toxin binding by depolarization but has no effect on saxitoxin binding. However, both intact and lysed synaptosomes bind to an epitope that is equally available in intact and lysed synaptosomes. This epitope is therefore present on the extracellular surface of the native sodium channel. This represents the first negatively charged site identified on the extracellular surface of

the channel and it is therefore a prime candidate for a neurotoxin receptor site. These antibodies do not affect [^{3}H]saxitoxin binding to sodium channels indicating that this epitope does not form part of neurotoxin receptor site 1. Similarly, they have no effect on neurotoxin binding and action at neurotoxin receptor sites 2 and 3.

We have now synthesized 12 more sodium channel peptides, purified and characterized them, coupled them to bovine serum albumin, and raised polyclonal antisera in multiple rabbits. These antibodies are now under intense study. All of them have affinity for the peptide against which they were raised. However, only 4 of these 12 antisera have measurable affinity for native sodium channels (Table 4). The remainder of these antibodies must be against regions of the sodium channel that are inaccessible in the native protein or are constrained in secondary structures that are not recognized by our antibodies. While the antibodies that do not interact with the native sodium channel will not be useful as agents to block neurotoxin action, we expect that they will be valuable reagents in mapping neurotoxin binding sites in defined regions of the channel structure.

TABLE I

Synergistic Effects of PbTx-2 and LqTx on BTX-B Binding to Sodium Channels

Condition	Specifically Bound [^{3}H]BTX-B (fmol)
Control	3.0
1.0 μM PbTx-2	9.6
1.0 μM LqTx	19.7
1.0 μM PbTx-2 + 1.0 μM LqTx	35.3

TABLE II

Neurotoxin Receptor Sites Associated with the Sodium Channel

Receptor Site*	Ligands	Physiological Effects
1	Tetrodotoxin Saxitoxin Geographutoxin II and other μ-conotoxins†	Inhibit ion conductance
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Persistent activation
3	α-Scorpion toxin Sea anemone toxin	Inhibit inactivation
4	β-Scorpion toxins	Shift activation
5	Brevetoxins	Shift activation Inhibit inactivation

* The sodium channel also has additional receptor sites for local anesthetics, certain anticonvulsants, pyrethroid insecticides, and coral toxins. However, since these receptor sites have not been directly identified in ligand binding studies, they are not listed here.

† Shown to act at neurotoxin receptor site 1 in references 26-28.

TABLE III

Representative Data on Monoclonal Antibodies against Rat Brain Na Channels

Antibody	Antigen Form	Antibody Titer (μM)	Antibody Intact (nM)	Affinity Solubilized (nM)	Inhibition of Toxin Binding		
					Site 1	Site 2	Site 3
2E8	Solublized	68	25	6.8	-	-	-
1A9	Solublized	18	15	3.6	-	-	-
1G11	Reconstituted	32		5.6	-	-	-
3B2	Reconstituted	7.5	1.8	0.8	-	-	-
3G4	Reconstituted	11	3.5	1.1	-	-	-

TABLE IV

Site Directed Antibodies

Synthetic Peptide	Reactivity with Peptide	Reactivity with Sodium Channel
1	+++	+++
2	+++	-
3	++	-
4	+++	-
5	++	-
6	++	±
7	++	-
8	+	-
9	++	±
10	+	-
11	+	+
11a	++	+
12	++	+
13	++	

FIGURE 1

Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Arg-Lys-Cys-Lys-

Asp-Arg-Arg-Cys-Lys-Hyp-Met-Lys-Cys-Cys-Ala-NH_2

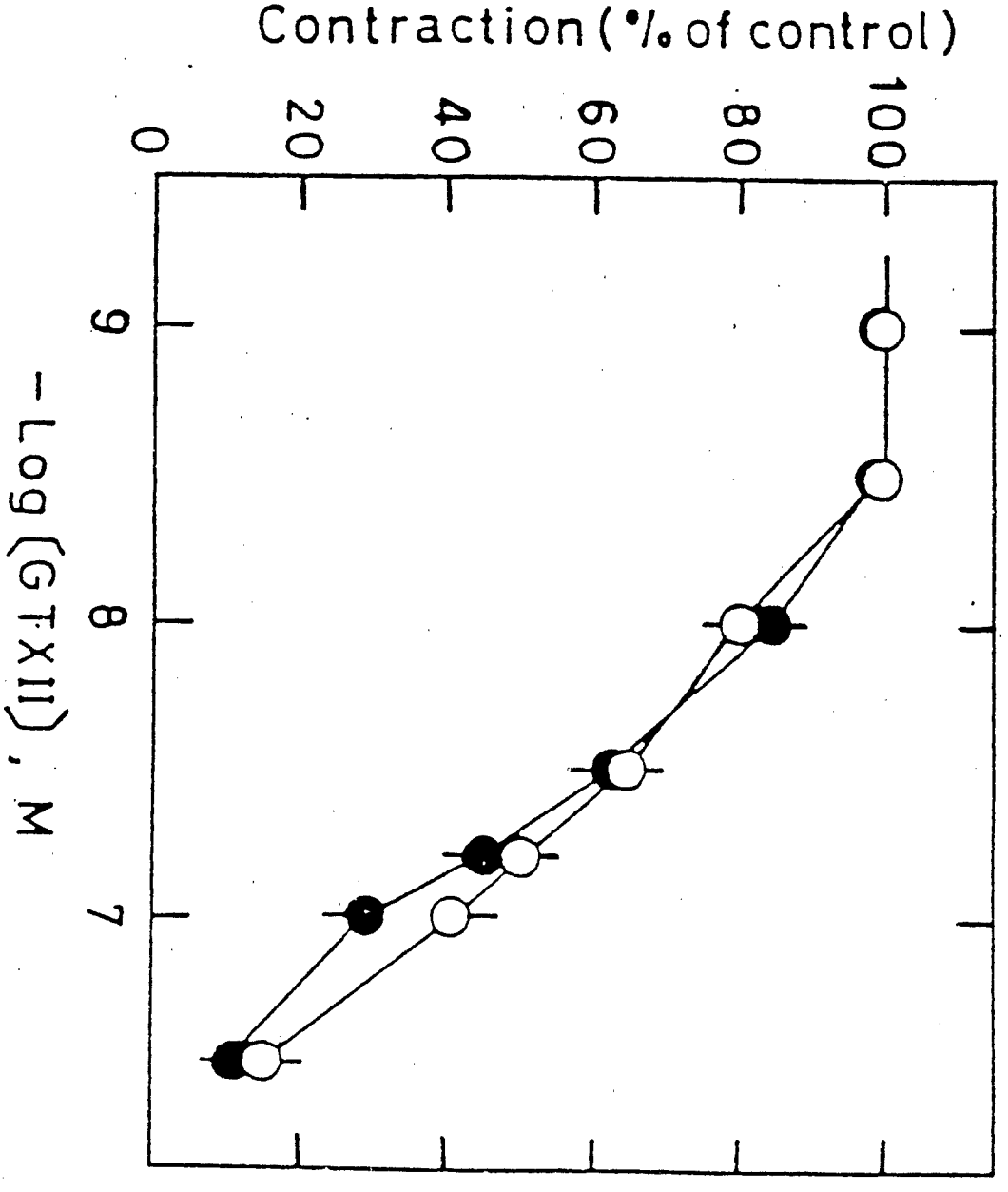


FIGURE 2

FIGURE 3

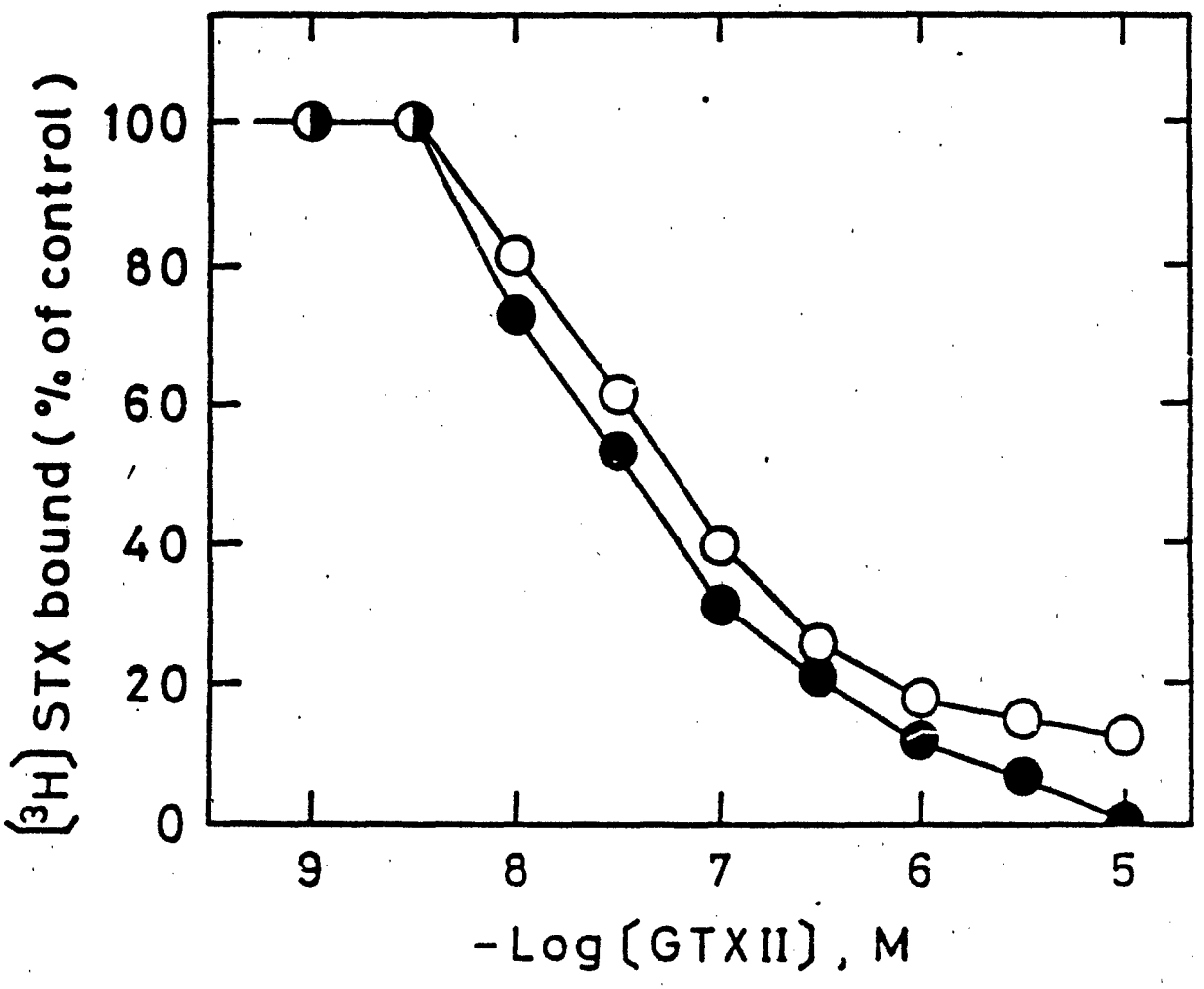


FIGURE 4

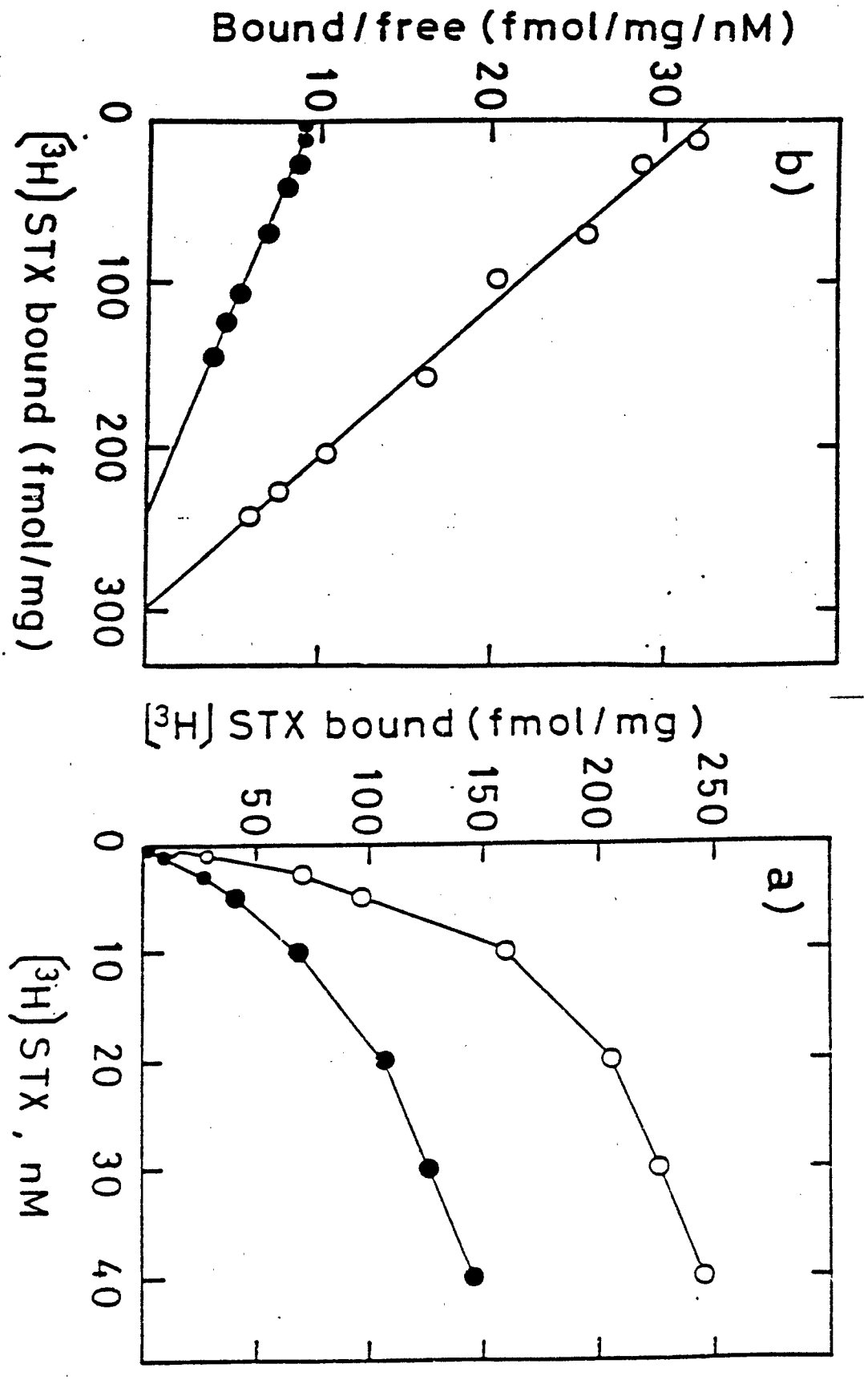

Bound/free (fmol/mg/nM)
10
20
30
0
100
200
300
[3H] STX bound (fmol/mg)
b)
[3H] STX bound (fmol/mg)
50
100
150
200
250
0
10
20
30
40
[3H] STX, nM
a)

FIGURE 5

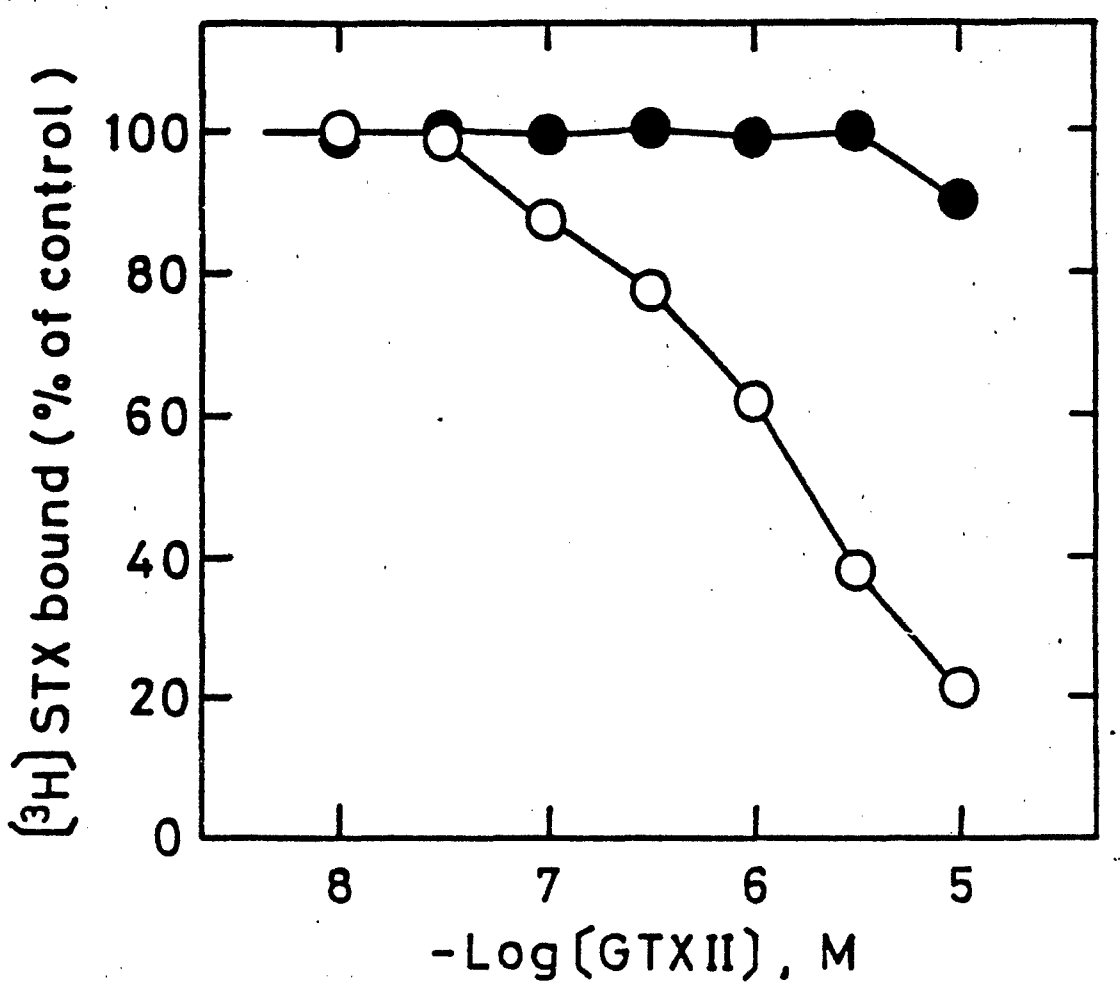


FIGURE 6

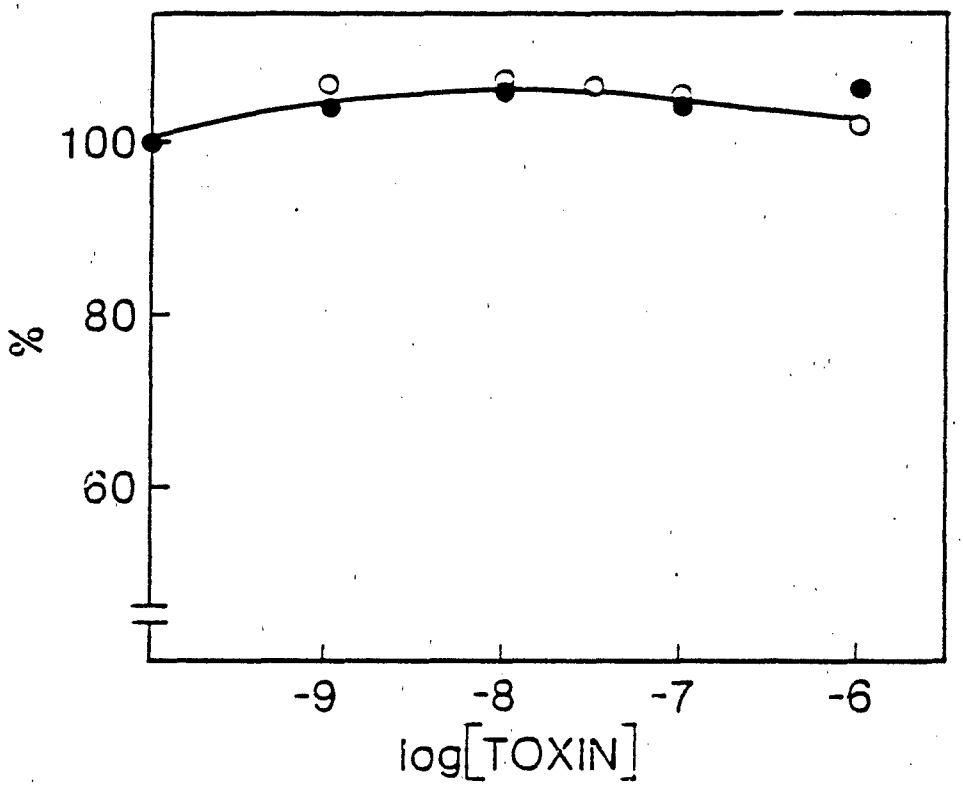


FIGURE 7

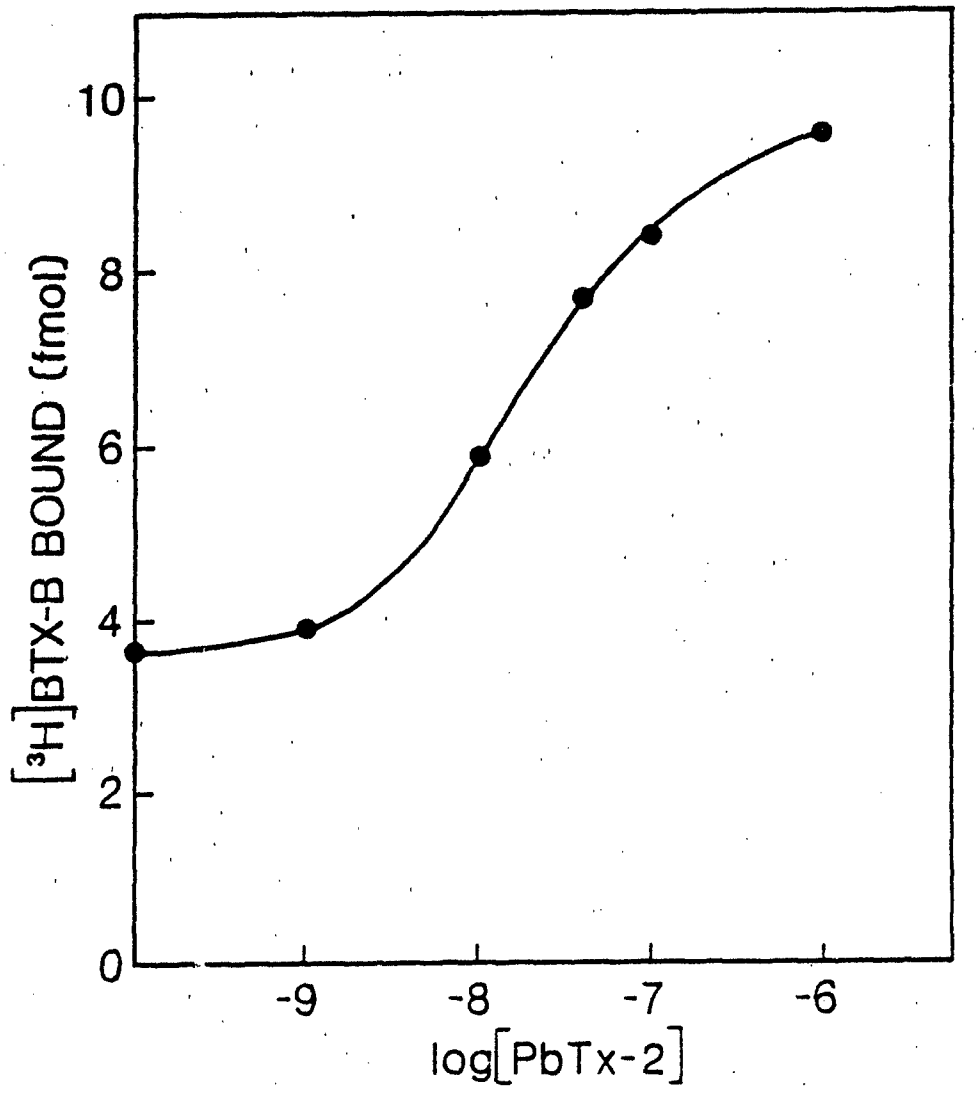


FIGURE 8

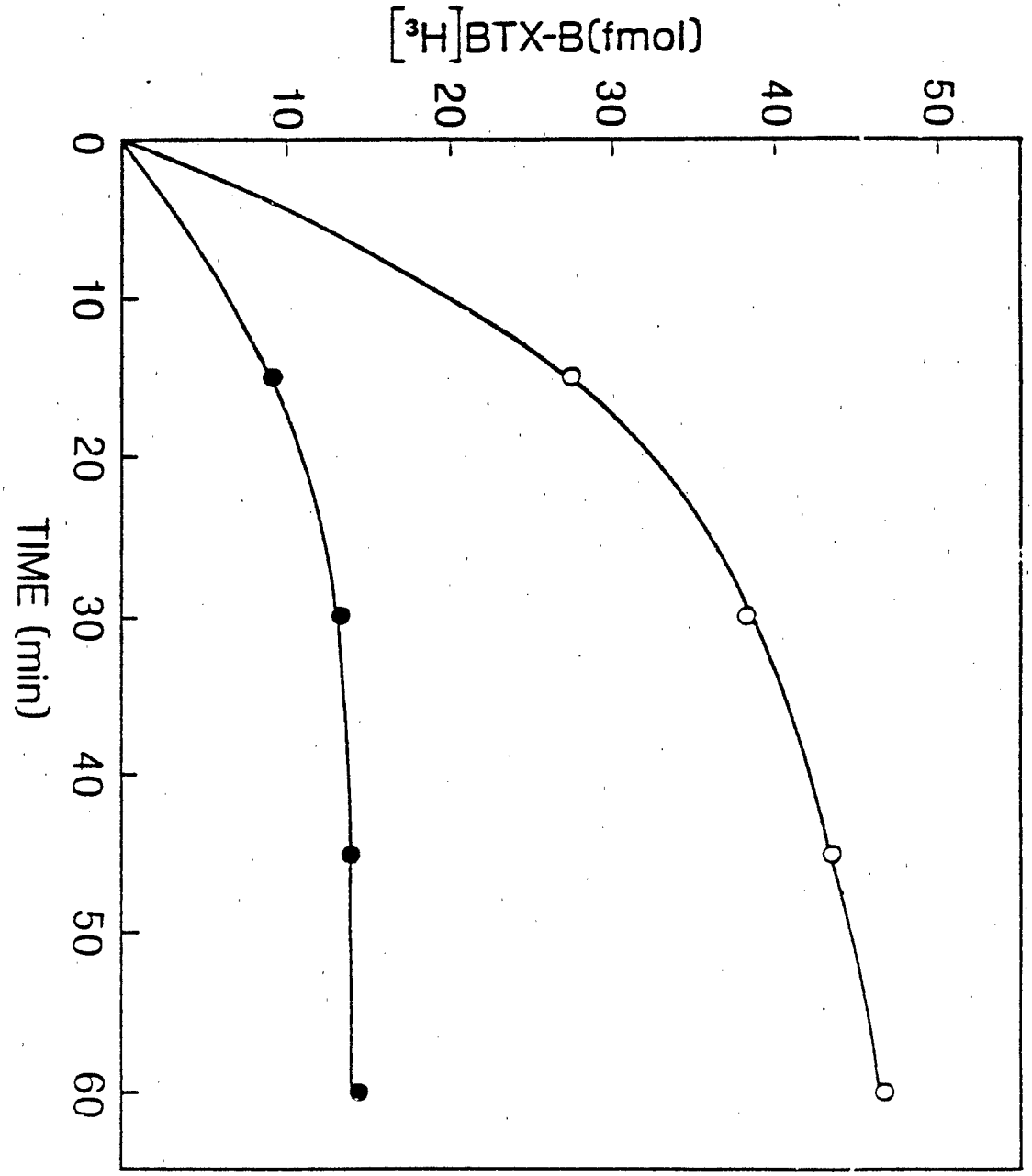


FIGURE 9

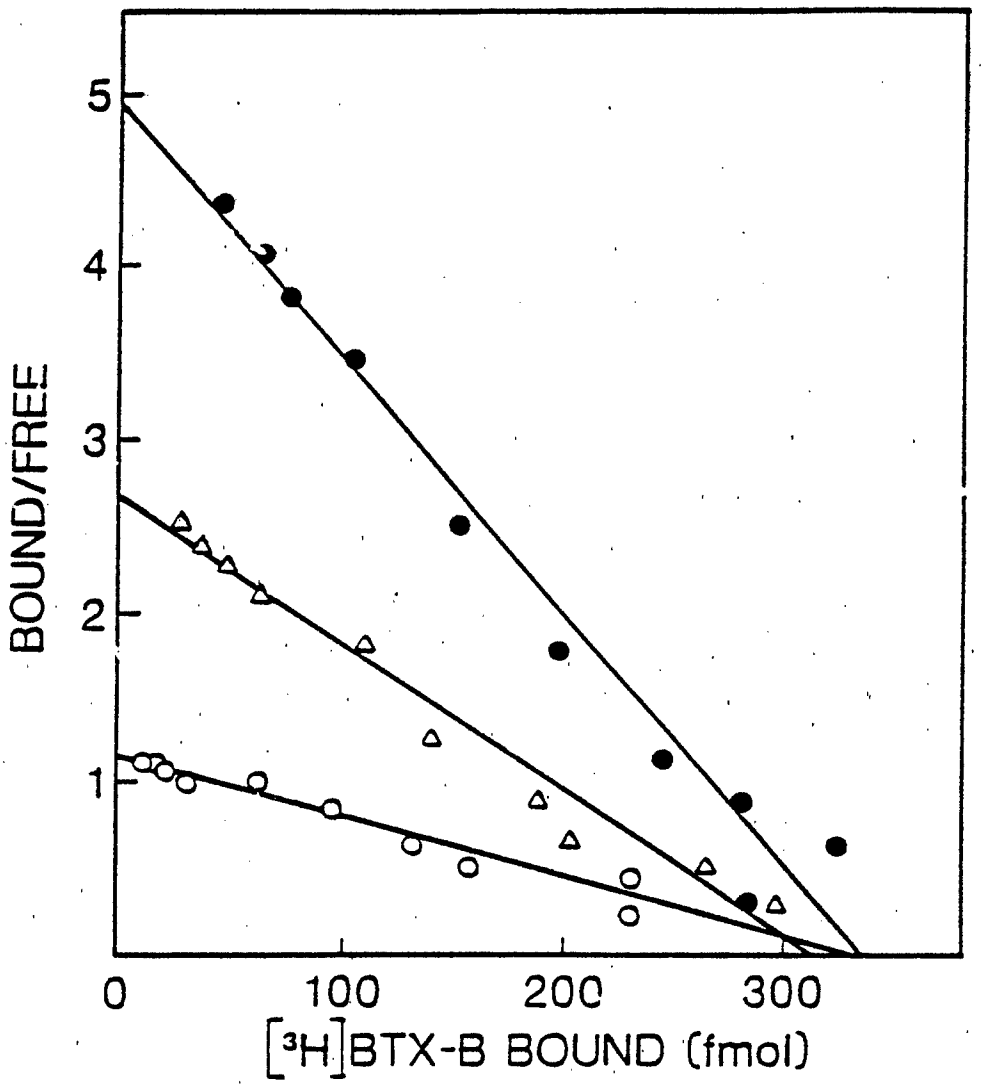


FIGURE 10

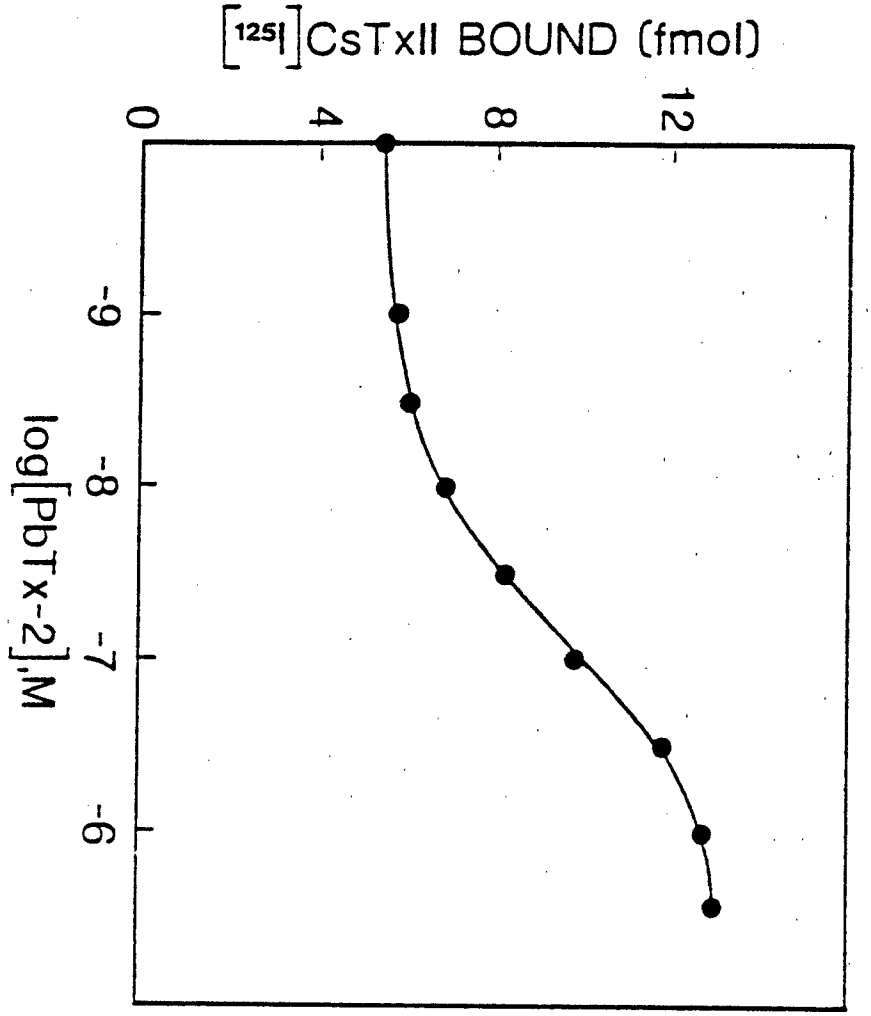
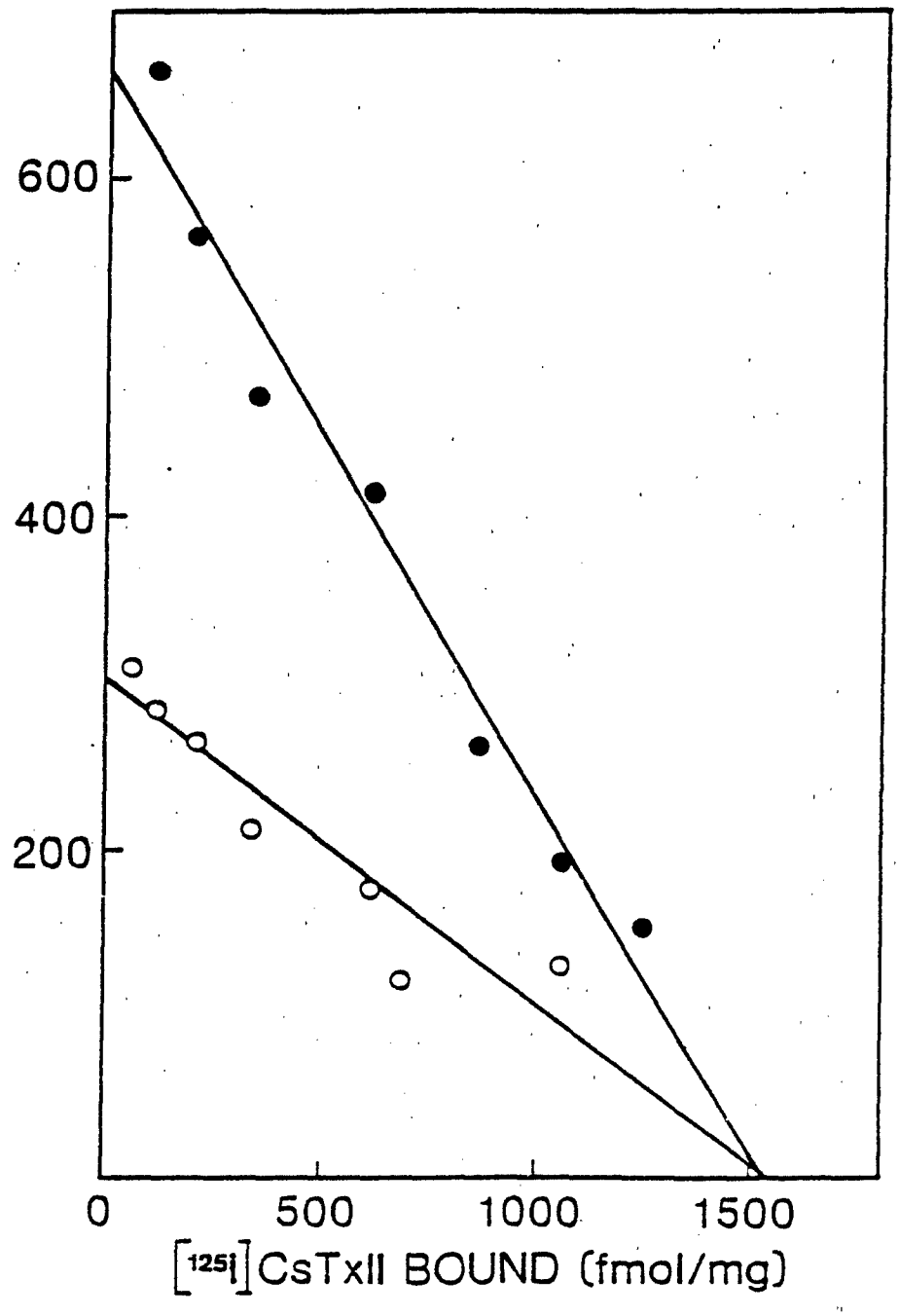


FIGURE 11.



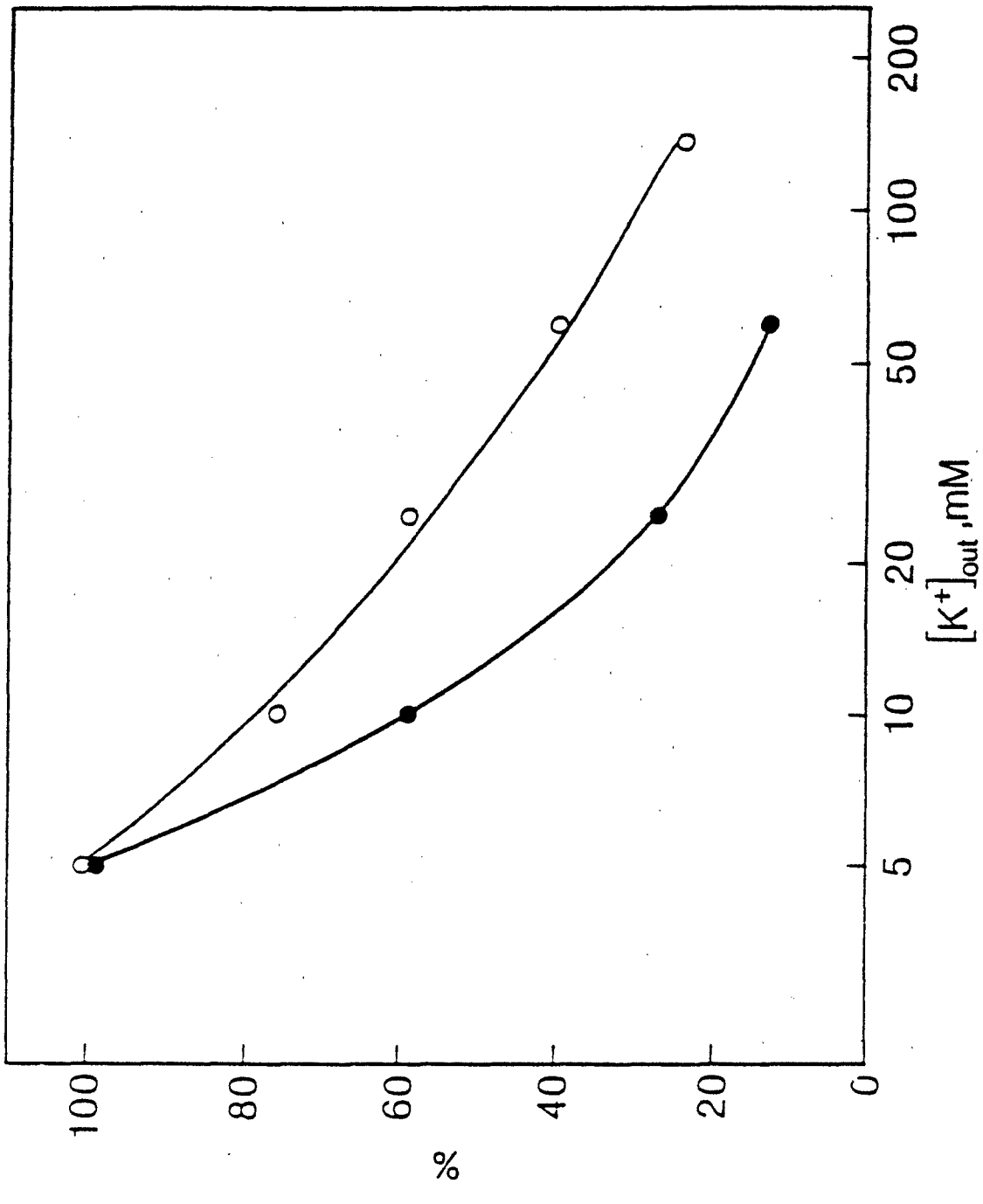


FIGURE 12

FIGURE 13

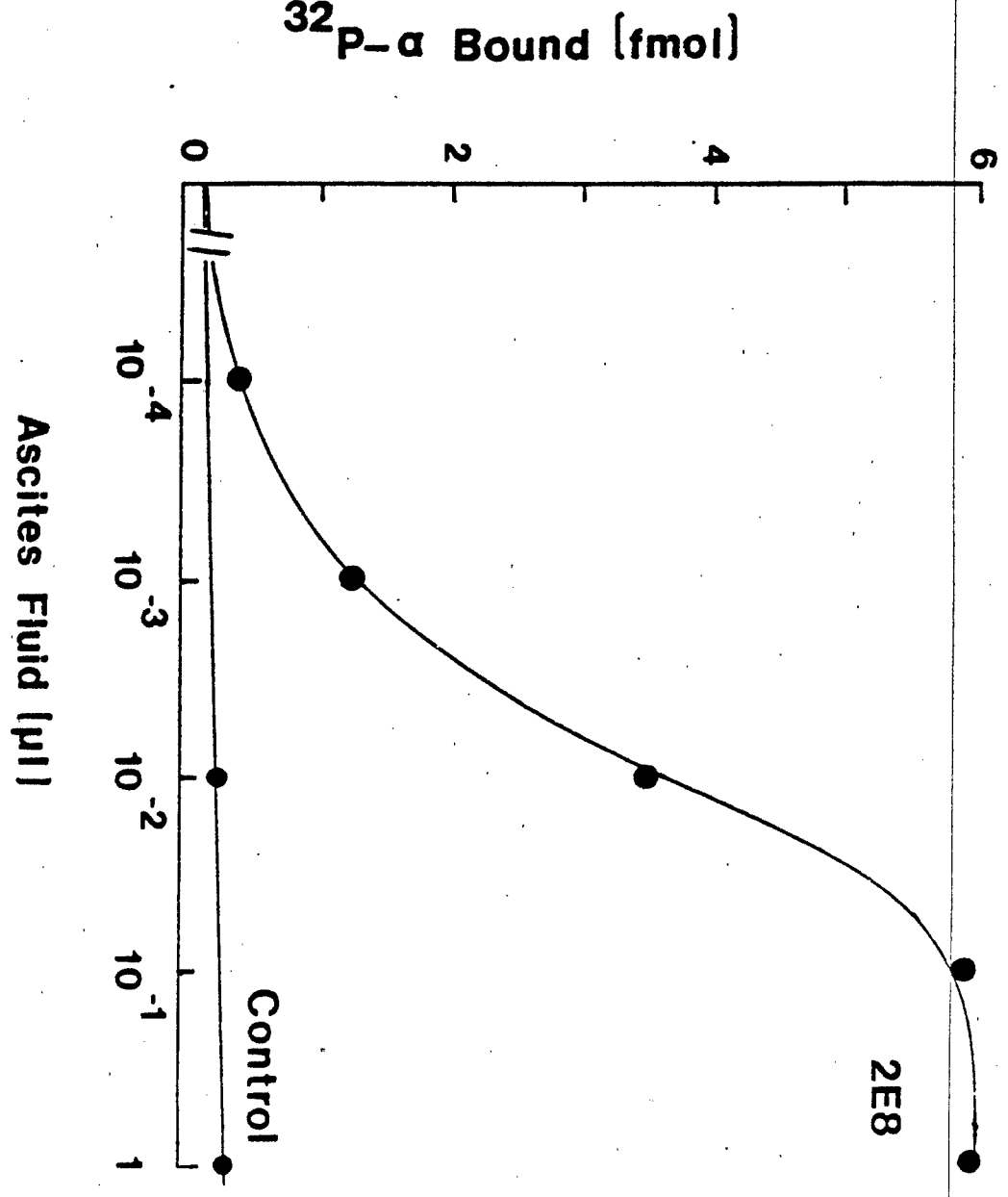

^{32}P–α Bound (fmol)
0
2
4
6
Ascites Fluid (µl)
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
2E8
Control

FIGURE 14A

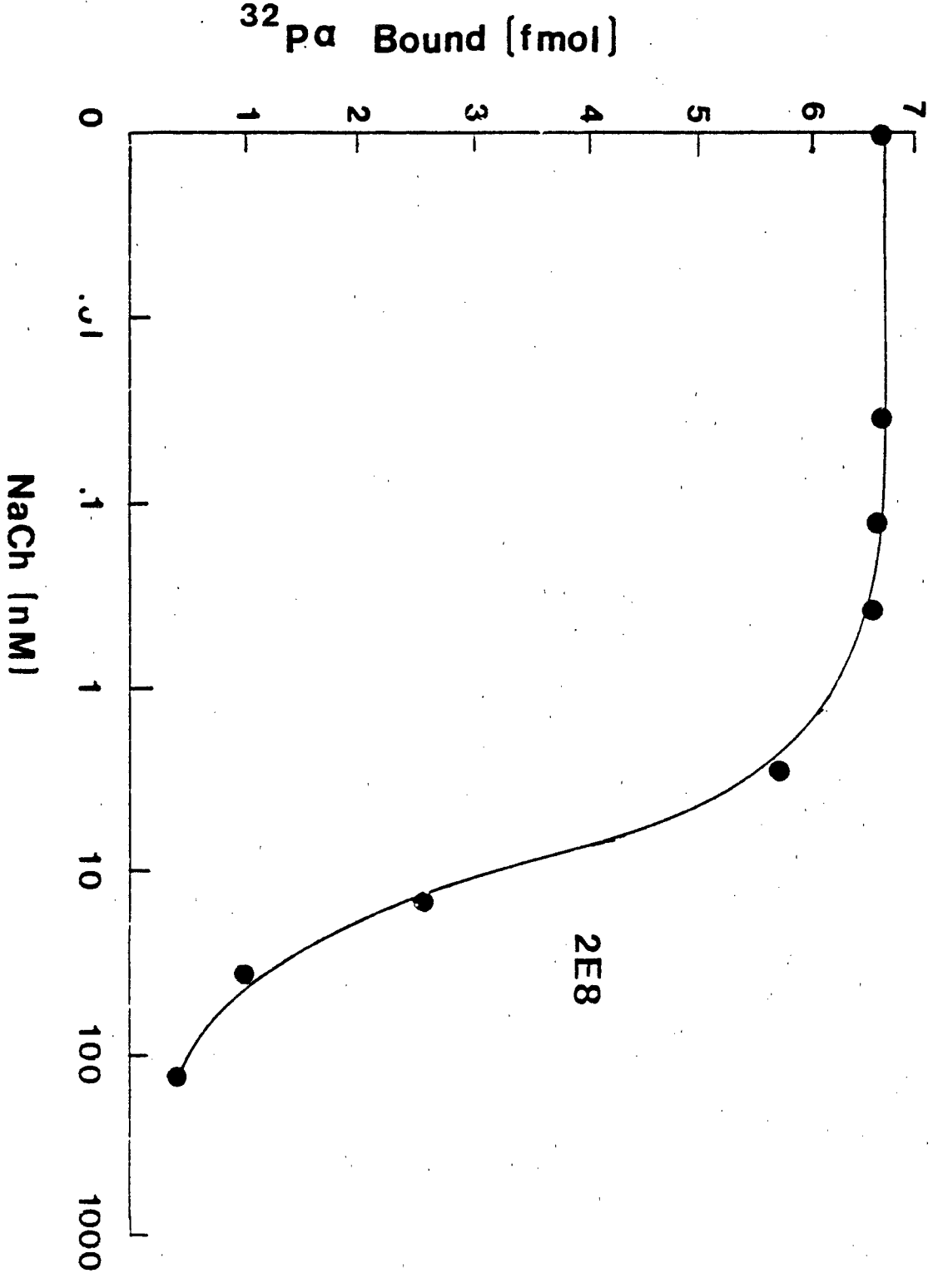


FIGURE 14B

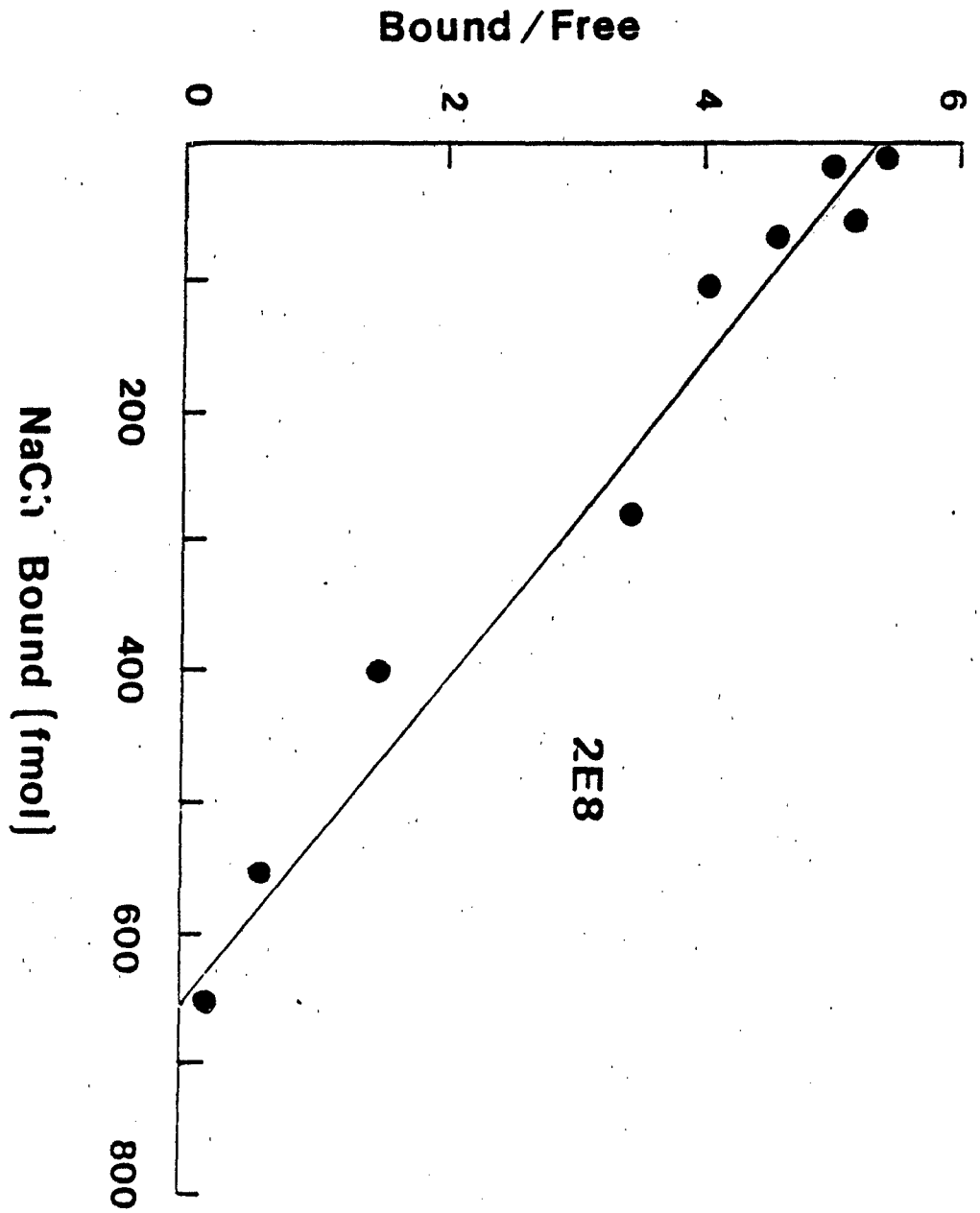
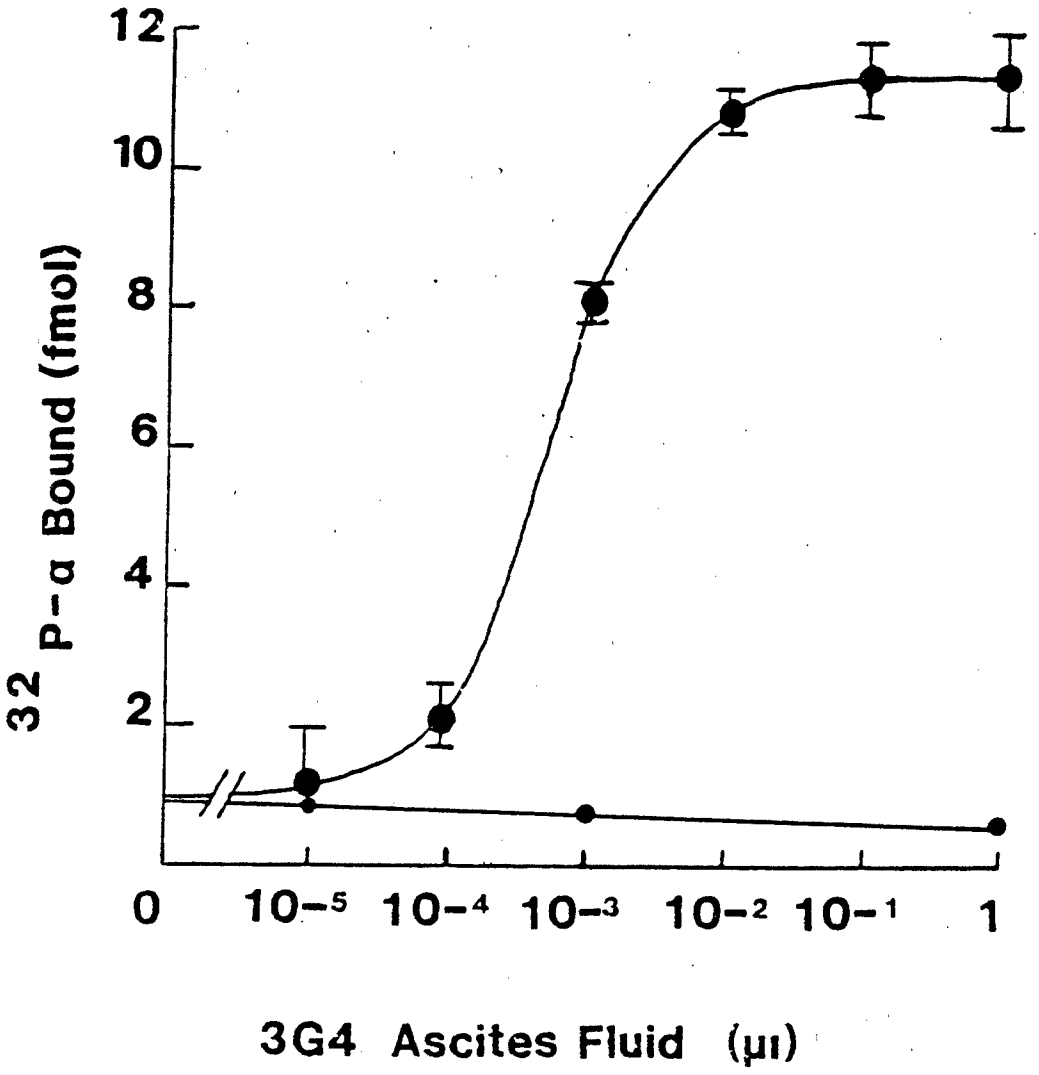


FIGURE 15


12
10
8
6
4
2
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
^{32}P-α Bound (fmol)
3G4 Ascites Fluid (μl)

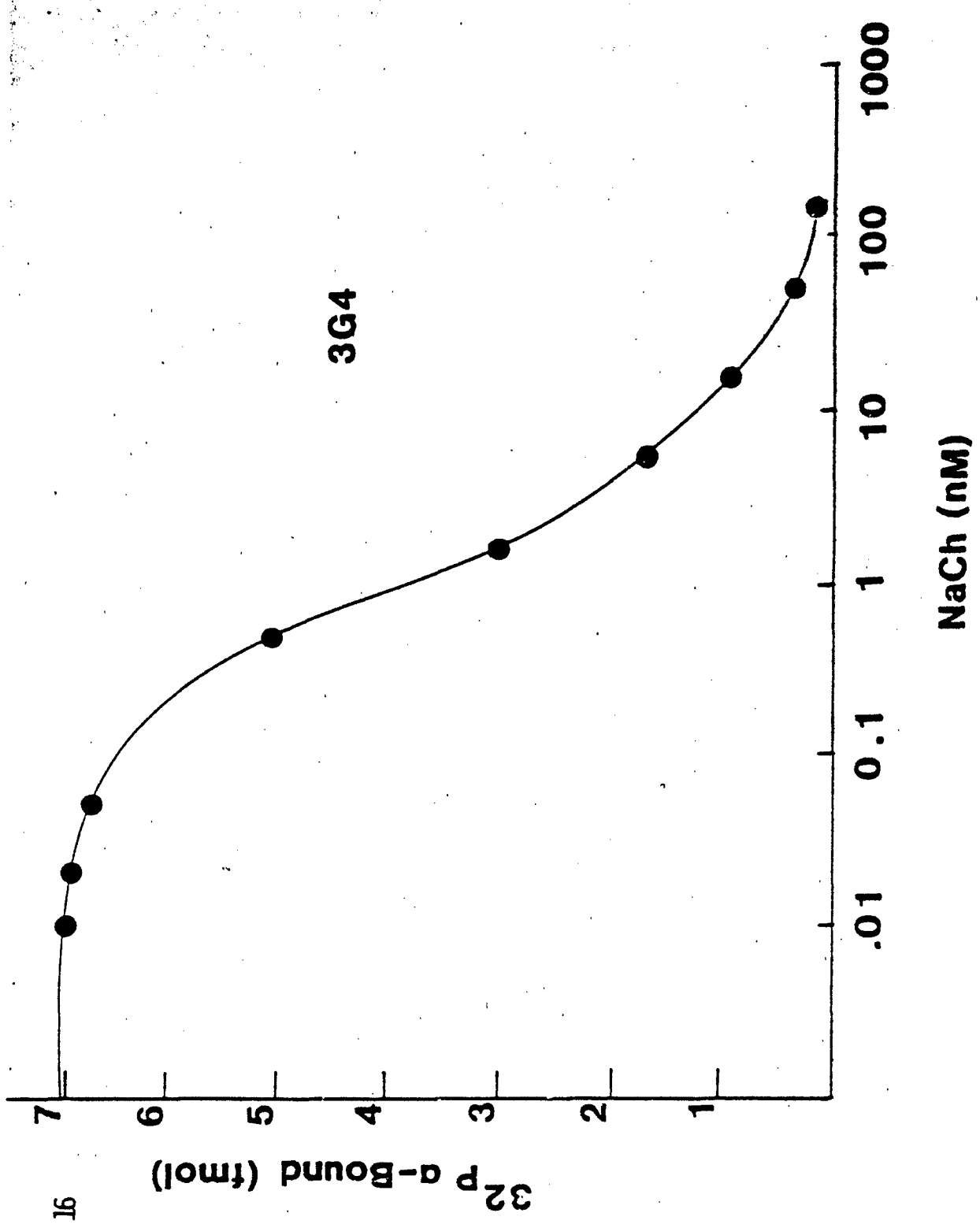
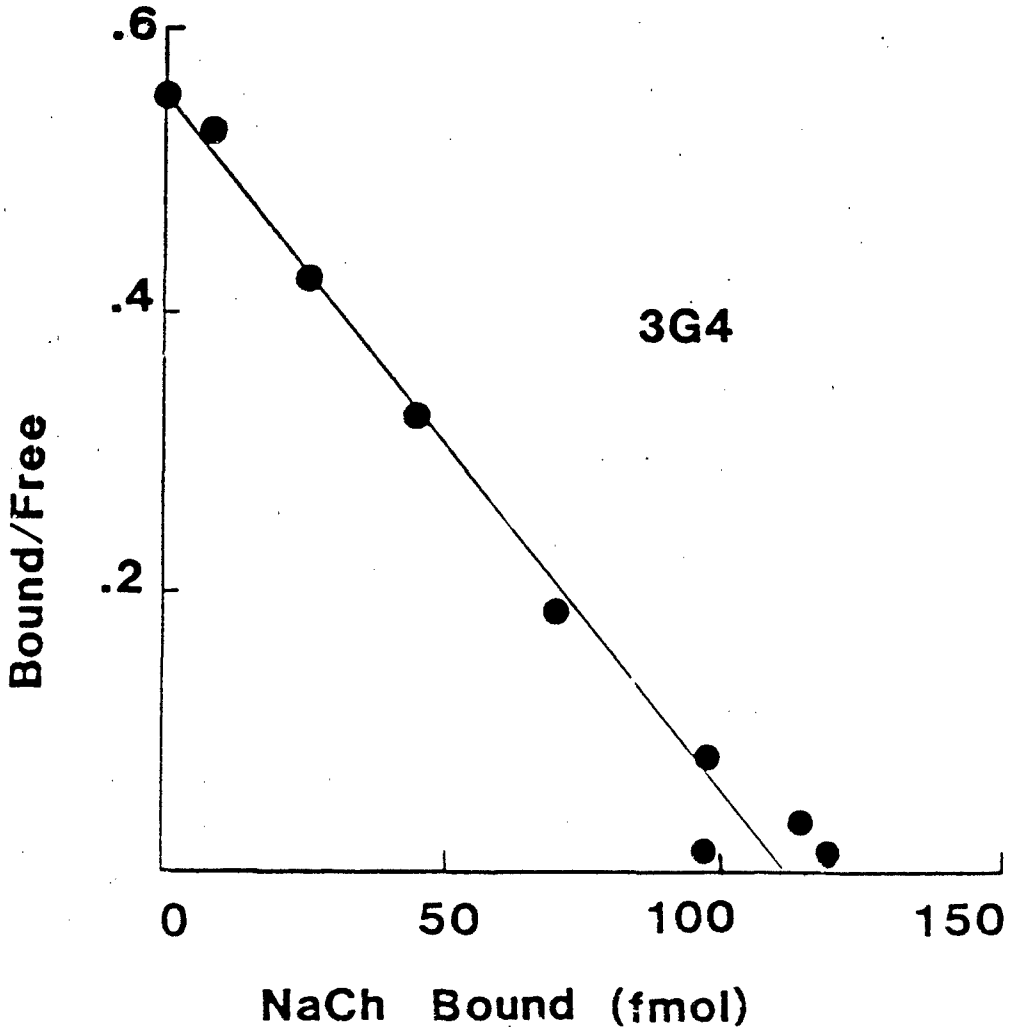


FIGURE 16

FIGURE 17



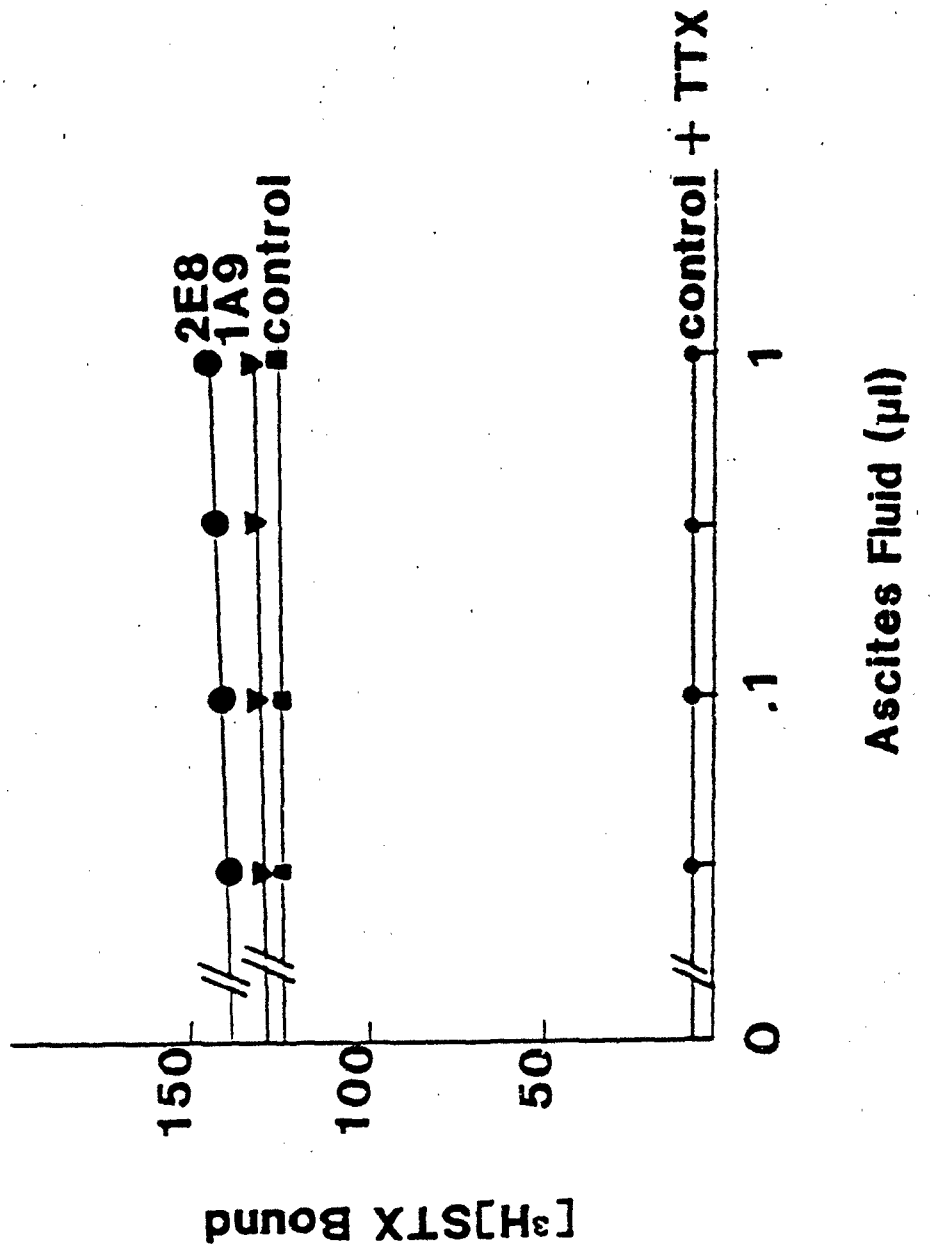

[3H]STX Bound
150
100
50
0
.1
1
Ascites Fluid (µl)
control + TTX
2E8
1A9
control

FIGURE 18A

FIGURE 18B

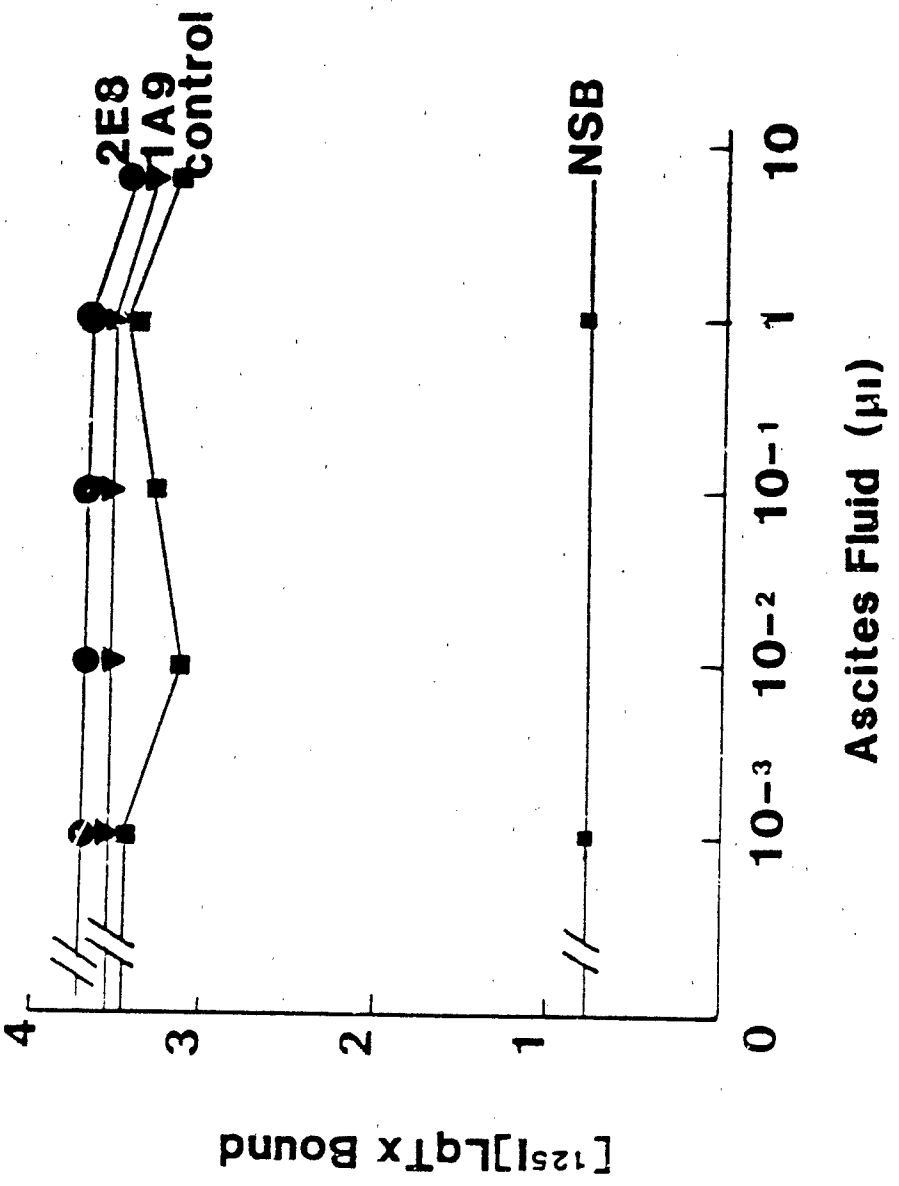

2E8
1A9
control
NSB
[125I]LqTx Bound
4
3
2
1
0
10-3
10-2
10-1
1
10
Ascites Fluid (µl)

FIGURE 19

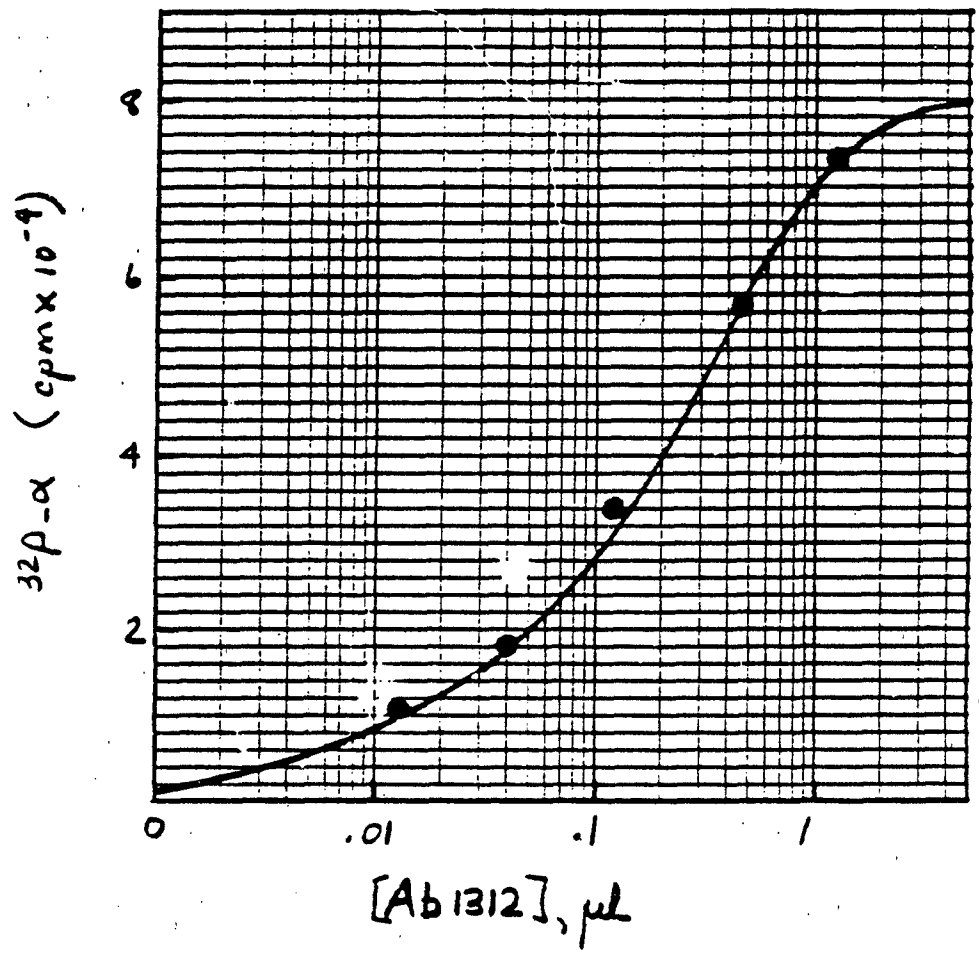


FIGURE 20

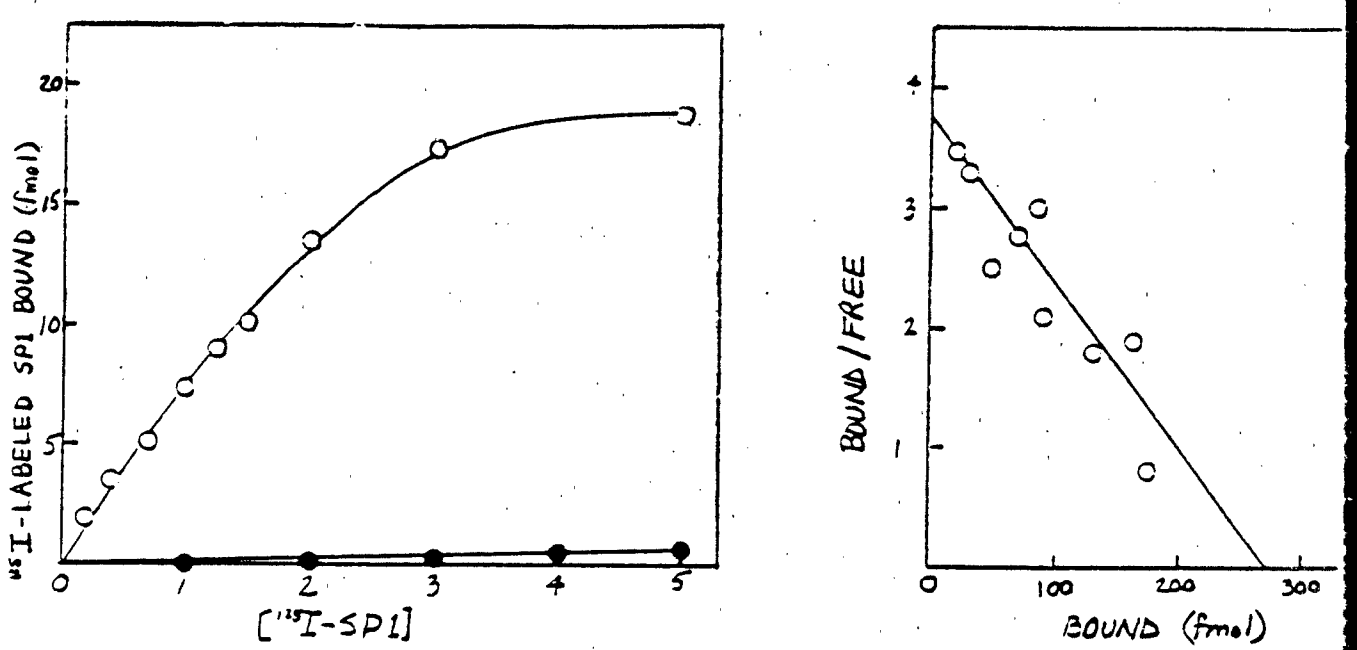

BINDING OF ^{125}I-LABELED SP1 BY Ab 1312
^{125}I-LABELED SP1 BOUND (fmol)
20
15
10
5
0
1
2
3
4
5
[^{125}I-SP1]
BOUND / FREE
4
3
2
1
0
100
200
300
BOUND (fmol)

FIGURE 21

BINDING OF SODIUM CHANNELS BY Ab 1312

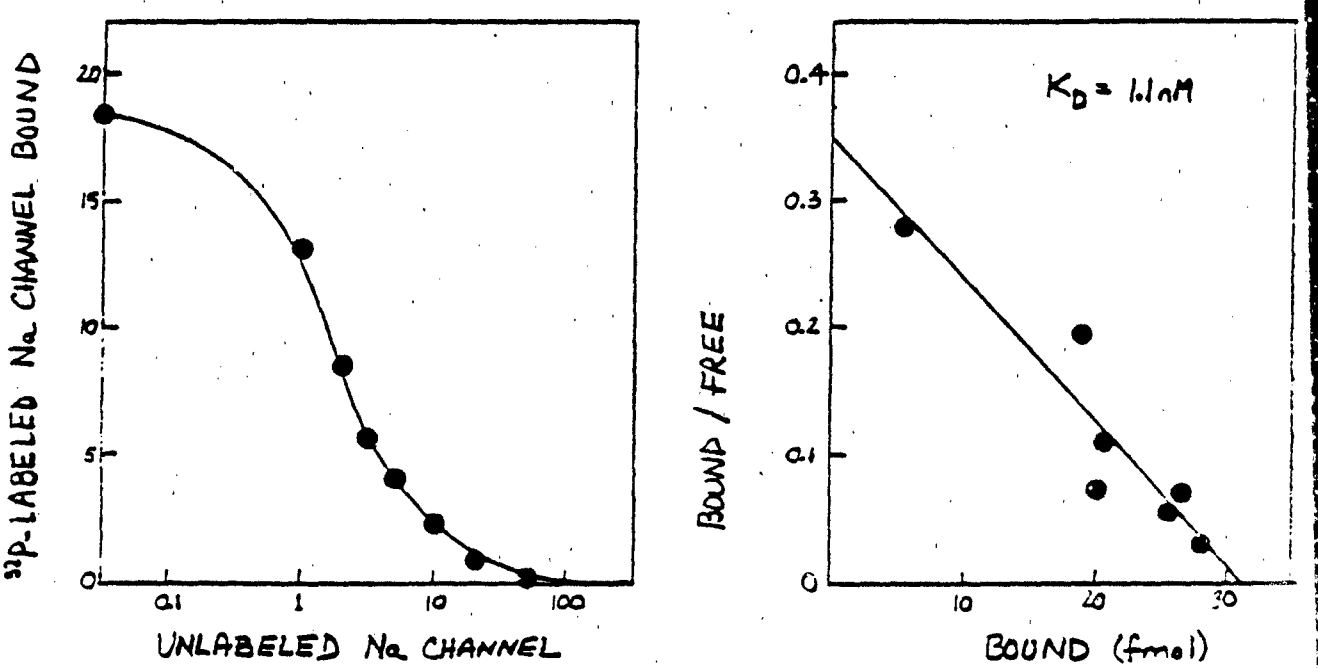


FIGURE 22

BINDING OF Ab 1312 TO SYNAPTOSOMES

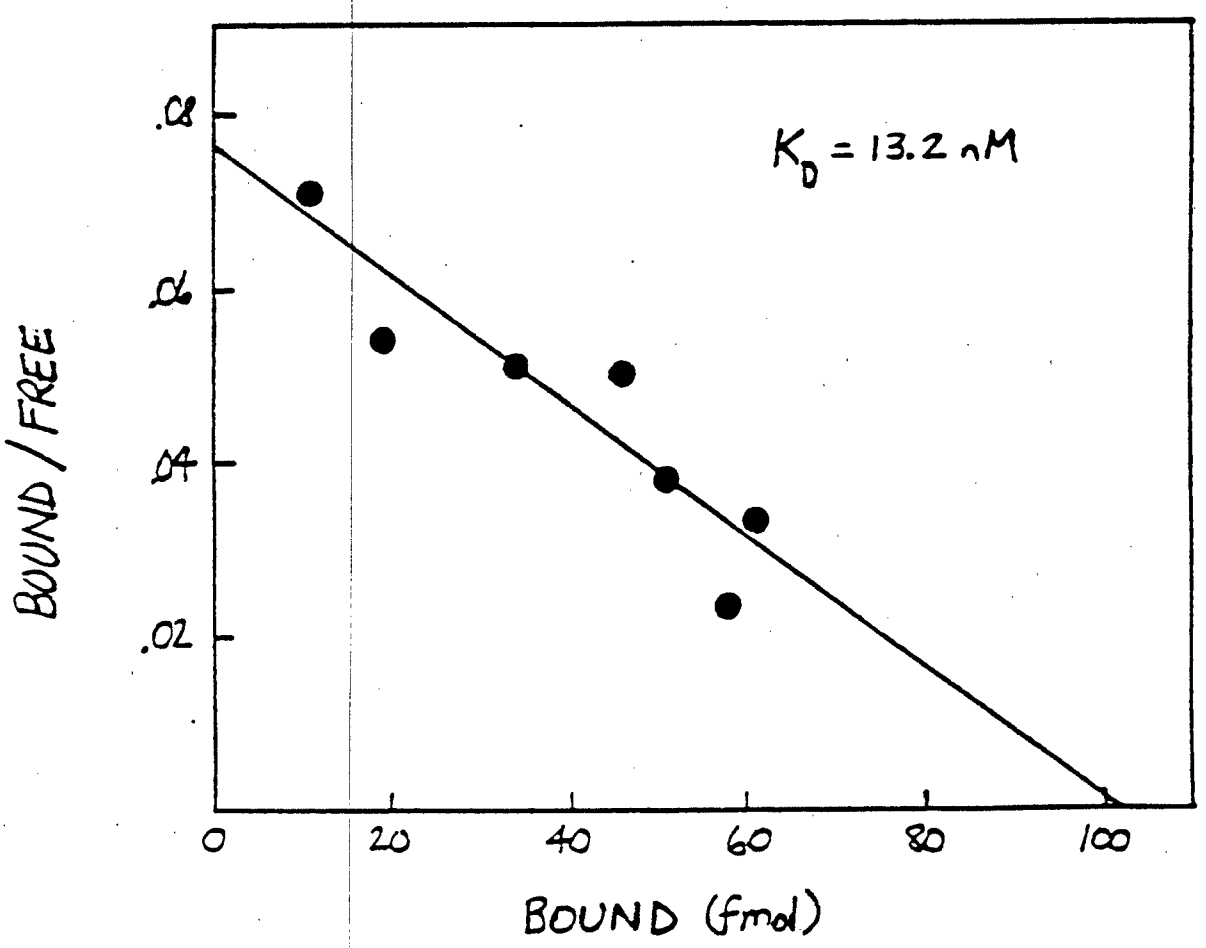
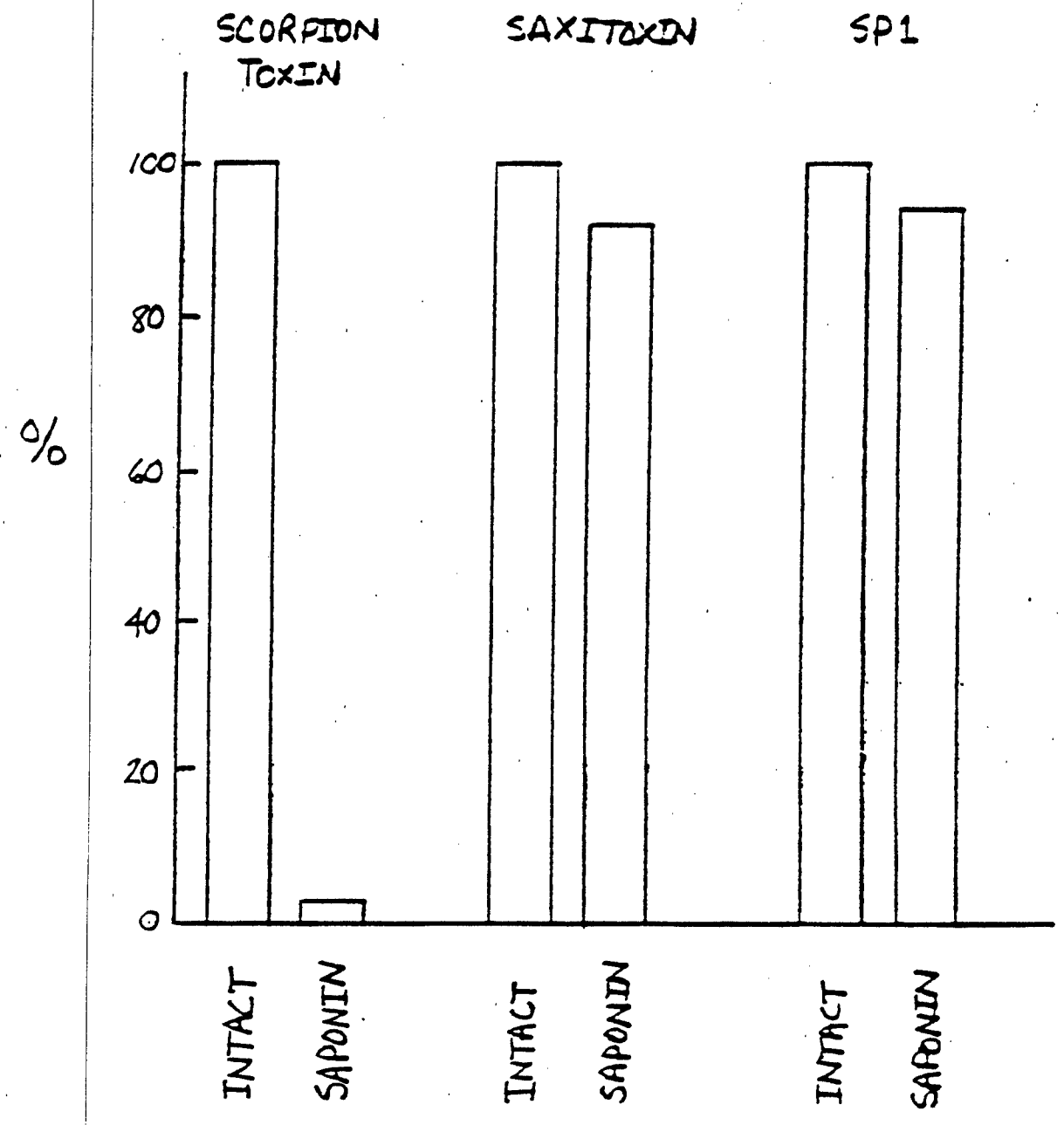


FIGURE 23

BINDING TO INTACT AND LYSED SYNAPTOSOMES



Legends for Figures

Figure 1. Amino acid sequence of GTX II.

Figure 2. Effects of GTX II on contractile responses of the isolated rat and rabbit diaphragm to direct electrical stimulation (0.1 Hz, 5 msec, supramaximal voltage). The twitch responses of rat (○) and rabbit (●) diaphragms were measured 30 min after exposure to different concentrations of GTX II. Each point on the graph represents the mean of 5 experiments. Vertical bars indicate standard error of the mean.

Figure 3. Effects of GTX II on [^{3}H]saxitoxin (STX) binding to rat skeletal muscle homogenates and T-tubular membranes of rabbit skeletal muscles. Muscle homogenates (○) or T-tubular membranes (●) were incubated with 5 nM [^{3}H]STX and the indicated concentrations of GTX II for 30 min at 36°C and bound STX was measured using the rapid membrane filtration assay. Nonspecific binding in the presence of 2 μM tetrodotoxin has been subtracted from the results.

Figure 4. Binding of [^{3}H]saxitoxin (STX) to T-tubular membranes of rabbit skeletal muscles in the presence or absence of GTX II. T-tubular membranes were incubated with increasing concentrations of [^{3}H]STX from 0.4 to 40 nM for 30 min at 36°C and bound STX was measured using the rapid membrane filtration assay. Nonspecific binding in the presence of 2 μM tetrodotoxin has been subtracted from the results. In (a), [^{3}H]STX binding was measured in the presence (●) or absence (○) of 0.5 nM GTX II and are plotted. In (b), [^{3}H]STX binding in the presence (●) or absence (○) of GTX II is presented as a Scatchard plot.

Figure 5. Effects of GTX II on [^{3}H]saxitoxin (STX) binding to rat brain synaptosomes and ganglion membranes. Brain synaptosomes (○) and ganglion membranes (●) were incubated with 2 nM [^{3}H]STX and the indicated concentrations of GTX II for 30 min at 36°C and bound STX was measured using the rapid membrane filtration assay. Nonspecific binding in the presence of 2 μM tetrodotoxin has been subtracted from the results.

Figure 6. Effect of PbTx-2 on specific binding of [^{3}H]STX and [^{125}I]$_1$LqTx to synaptosomes. Specific binding of 3 nM [^{3}H]STX (●) or 0.1 nM [^{125}I]$_1$LqTx (○) to synaptosomes was measured as described under Experimental Procedures in the presence of the indicated concentrations of PbTx-2.

Figure 7. Enhancement of specific binding of [^{3}H]BTX-B to synaptosomes by PbTx-2. Specific binding of 10 nM [^{3}H]BTX-B to synaptosomes was measured as described under Experimental Procedures in the presence of the indicated concentrations of PbTx-2.

Figure 8. Time course of specific binding of [^{3}H]BTX in the presence of PbTx-2 or PbTx-2 and LqTx. Specific binding of 10 nM [^{3}H]BTX-B to synaptosomes was measured in the presence of 1 μM PbTx-2 (●) or 1 μM PbTx-2 plus 1 μM LqTx (○) after incubation for the indicated time at 37°C as described under Experimental Procedures.

Figure 9. Scatchard analysis of specific binding of [^{3}H]BTX-B in the presence of LqTx and PbTx. Specific binding of 10 nM to 1.0 μM [^{3}H]BTX-B to synaptosomes was measured in the presence of 1 μM PbTx-2 (○), 1 μM LqTx (△), or 1 μM PbTx-2 plus 1 μM LqTx (●) was measured as described under Experimental Procedures. Ordinate: fmol/nM.

Figure 10. Enhancement of specific binding of 0.25 nM [^{125}I]CsTx II to synaptosomes was measured in the presence of the indicated concentrations of PbTx-2 as described under Experimental Procedures.

Figure 11. Scatchard analysis of specific binding of [^{125}I]CsTx II to synaptosomes in the presence of PbTx-2. Specific binding of 0.25 nM to 8.0 nM [^{125}I]CsTx II to synaptosomes was measured in the absence (○) or presence (●) of 1 μM PbTx-2 as described in Experimental Procedures. Ordinate: fmol/nM.

Figure 12. Voltage dependence of enhancement of [^{3}H]BTX-B binding by PbTx-2. Specific binding of 10 nM [^{3}H]BTX-B to synaptosomes was measured in the presence of 30 nM PbTx-2 and the indicated concentrations of K^+ as described under Experimental Procedures (○). KCl was exchanged for an equal concentration of choline chloride in standard binding medium. Binding of [^{3}H]BTX-B in the absence of PbTx-2 was subtracted from the data. For comparison, specific binding of 0.2 nM [^{125}I]$_1$LqTx to synaptosomes was measured under the same conditions as described under Experimental Procedures (●).

Figure 13. Radioimmune assay of mAb 2E8 against detergent-solubilized sodium channels. The indicated volumes of ascites fluid by hybridoma 2E8 were incubated with 0.1 nM ^{32}P-labeled sodium channels and antibody-bound sodium channels were precipitated by adsorbtion to protein A-Sepharose.

Figure 14. Scatchard analysis of binding of mAb 2E8. A. A fixed concentration of mAb 2E8 was incubated with 0.1 nM ^{32}P-labeled sodium channels and the indicated concentrations of unlabeled sodium channels. Antibody-bound, ^{32}P-labeled sodium channels were precipitated by adsorbtion to protein A-Sepharose. B. The data of panel A were analyzed according to Scatchard. The best fit regression line is drawn for a K_D of 6.4 nM and B_{max} of 6.8 μM.

Figure 15. Radioimmune assay of mAb 3G4 against reconstituted sodium channels. The indicated volumes of ascites fluid produced by hybridoma 3G4 were incubated with 0.1 nM ^{32}P-labeled sodium channels and antibody-bound sodium channels were precipitated by adsorption to protein A-Sepharose.

Figure 16. Affinity of mAb 3G4 for sodium channels in synaptosomes. A fixed concentration of mAb 3G4 was incubated with increasing concentrations of synaptosomal sodium channels. Synaptosomes and bound mAb's were removed from the sample by centrifugation in an airfuge. mAb's remaining in the supernatant were then assayed by radioimmune assay: the supernatants were incubated with 0.1 nM ^{32}P-labeled sodium channels and antibody-bound sodium channels were precipitated by adsorbtion to protein A-Sepharose.

Figure 17. Scatchard analysis of mAb 3G4 binding. The data in Figure 16 are plotted as a Scatchard plot. The regression line corresponds to K_D = 1.1 nM and B_{max} = 11 μM.

Figure 18. Effect of mAb's on binding of saxitoxin and scorpion toxin. A. Saxitoxin binding to sodium channels in synaptosomes was measured as previously described (29) in the presence of the indicated amounts of mAb's or control preimmune serum. B. Binding of the α-scorpion toxin from Leiurus quinquestriatus to sodium channels in synaptosomes was measured as previously described (29) in the presence of the indicated amounts of mAb's or control preimmune serum.

Figure 19. Radioimmune assay of antibody against peptide SP1. The indicated volumes of antiserum 1312 were incubated with 0.1 nM ^{32}P-labeled sodium channels and the antibody-bound channels were precipitated by adsorbtion to protein A-Sepharose.

Figure 20. Binding of SP1 to antibody 1312. A. ^{125}I-labeled SP1 was incubated at the indicated concentration (0-5 nM) with antiserum 1312 and the antibody-bound peptide was precipitated by adsorbtion to protein A-Sepharose. B. The data of panel A are present as a Scatchard plot. The regression line gives a K_D of 0.8 nM.

Figure 21. Scatchard analysis of binding of sodium channels by antibody against SP1. The experiment was carried out as described in the legend to Figure 16. The regression line gives a K_D of 1.1 nM.

Figure 22. Binding of antibody against SP1 to sodium channels in synaptosomes. The experiment was performed as described in the legend to Figure 17. The regression line gives a K_D of 13.2 nM.

Figure 23. Effect of lysis with saponin on binding of antibody against SP1 to sodium channels in synaptosomes. Synaptosomes were prepared as described previously (29). Part of the preparation was lysed by treatment with 0.2% saponin. Specific binding of [^{3}H]saxitoxin and ^{125}I-labeled scorpion toxin was measured for intact and lysed synaptosomes as described previously (29). The IgG fraction of antiserum 1312 was labeled with ^{125}I by the chloramine T method. The labeled antibodies were incubated with synaptosomes for 16 hr at 4° and synaptosomes with bound antibodies were isolated by sedimentation in a microfuge and washing.

References

1. Kohn, A. J. (1958) Hawaii Med. J. 17:528-532.
2. Whyte, J. and Endean, R. (1962) Toxicon 1:25-31.
3. Endean, R., Parish, G. and Gyr, P. (1974) Toxicon 12:131-138.
4. Kobayashi, J., Nakamura, H., Hirata, Y. and Ohizumi, Y. (1982) Toxicon 20:823-830.
5. Gray, W.R., Luque, A., Olivera, B.M., Barrett, J. and Cruz, L.J. (1981) J. Biol. Chem. 256:4734-4740.
6. Olivera, B.M., McIntosh, M., Cruz, L.J., Luque, F.A. and Gray, W.R. (1984) Biochemistry 23:5087-5090.
7. Kerr, L.M. and Yoshikami, D. (1984) Nature 308:282-284.
8. Nakamura, H., Kobayashi, J., Ohizumi, Y. and Hirata, Y. (1983) Experientia 39:590-591.
9. Sato, S., Nakamura, H., Ohizumi, Y., Kobayashi, J. and Hirata, Y. (1983) FEBS Lett. 155:277-280.
10. Cruz, L.J., Gray, W.R., Olivera, B.M. Zeikus, R.D., Kerr, L., Yoshikami, D. and Moczydlowski, E. (1985) J. Biol. Chem. 260:9280-9288.
11. Minoshima, S., Kobayashi, M., Nakamura, H., Kobayashi, J., Ogura, A., Takahashi, M. and Ohizumi, Y. (1984) Jpn. J. Pharmacol. 36:192P.
12. Ohizumi, Y., Nakamura, H. and Kobayashi, J. (1986) Eur. J. Pharmacol., in press.
13. Catterall, W.A. (1984) Science 223:653-661.
14. Ritchie, J.M. and Rogart, R.B. (1977) Rev. Physiol. Biochem. Pharmacol. 79:1-51.
15. Campbell, D.T. and Hille, B. (1976) J. Gen. Physiol. 67:309-323.
16. Chang, C.C. and Tseng, K.H. (1978) Br. J. Pharmacol. 63:551-559.
17. Woilner, D.A. and Catterall, W.A. (1985) Brain Res. 331:145-149.
18. Moczydlowski, E., Garber, S.S. and Miller C. (1984) J. Gen. Physiol. 84:665-686.
19. Risk, M., Lin, Y.Y., Ramanujam, V.M.S., Smith, L.L., Ray, S.M., and Trieff, N.M. (1979) J. Chromatograph Sci. 17:400-405.
20. Chou, H.N. and Shimizu, Y. (1982) Tetrahedron Lett. 23:5521-5524.

21. Shimizu, Y., Chou, N.M., Bando, H., VanDuyne, G. and Clardy, J. (1986) J. Am. Chem. Soc. **108**:514-515.

22. Baden, D.G., Mende, T.J. Lichter, W., Wellham, L.L. (1981) Toxicon **19**:455-462.

23. Lin Y.Y., Risk, M., Ray, S.M. VanEngen, D., Clardy, J., Golik, J., James, J.C. and Nakanishi, K. (1981) J. Am. Chem. Soc. **103**:6773-6774.

24. Baden, D.G. Mende, T.J. (1982) Toxicon **20**:457-461.

25. Catterall, W.A. and Risk, M.A. (1981) Mol. Pharmacol. **19**:345-348.

26. Catterall, W.A. and Gainer, M. Toxicon **23**:497-504.

27. Huang, J.M.C., Wu, C.H. and Baden, D.G. (1984) J. Pharmacol. Exp. Therapeut. **229**:615-621.

28. Catterall, W.A. (1980) Ann. Rev. Pharmacol. Toxicol. **20**:15-83.

29. Tamkun, M.M. and Catterall, W.A. (1981) Mol. Pharmacol. **19**:78-86.

30. Catterall, W.A., Morrow, C.S., Daly, J.W. and Brown G.B. (1981) J. Biol. Chem. **256**:8922-8927.

31. Jover, E., Couraud, F. and Rochat, H. (1980) Biochem. Biophys. Res. Comm. **95**:1607-1614.

32. Poli, M.A., Mende, T.J. and Baden, D.G. (1986) Mol. Pharmacol. **30**:129-135.

33. Catterall, W.A. (1977) J. Biol. Chem. **252**:8669-8676.

34. Moore, H., Fritz, L., Raftery, M. and Brockes, J. (1982) Proc. Natl. Acad. Sci. USA **79**:1673-1677.

35. Fritz, L and Brockes, J.P. (1983) J. Neurosci. **3**:2300-2309.

36. Meiri, H., Zeitoun, I., Grunhagen, H., Lev-Ram, V., Eshhar, Z. and Schlessinger, J. (1984) Brain Res. **310**:168-173.

37. Barhanin, J., Meiri, H., Romey, G., Pauron, D. and Lazdunski, M. (1985) Proc. Natl. Acad. Sci USA **82**:1842-1846.

38. Haimovich, B., Bonilla, E., Casadei, J. and Barchi, R. (1984) J. Neurosci. **4**:2259-2268.

39. Casadei, J.M., Gordon, R., Lampson, L., Schotland, D. and Barchi, R. (1984) **81**:6227-6231.

40. Wollner, D. and Catterall, W.A. (1985) Brain Res. **331**:145-149.

41. Costa, M. and Catterall, W.A. (1984) J. Biol. Chem. **259**:8210-8218.

42. Reading, C. (1982) J. of Immun. Methods **53**:261-291.

43. Auld, V., Marshall, J., Goldin, A., Dowsett, A., Catterall, W.A., Davidson, N. and R. Dunn. (1985) J. Gen. Physiol. 86:10a.

44. Catterall, W.A. (1986) Ann. Rev. Biochem. 55:953-985.

45. Noda, M., et al (1986) Nature 320:188-192.

46. Ohizumi, Y., Nakamura, H., Kobayashi, J., Catterall, W.A. (1986) J. Biol. Chem. 261:6149-6152.

47. Gonoi, T., Sherman, S.J., Catterall, W.A. (1985) J. Neurosci. 5:2559-2564.